University of Alberta

COX16 encodes a novel protein required for the assembly of cytochrome oxidase in Saccharomyces cerevisiae.

by

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Abstract

I have characterized Cox16p, a new cytochrome oxidase (COX) assembly factor. This protein is encoded by *COX16*, corresponding to the previously uncharacterized open reading frame, YJL003w, of the yeast genome. *COX16* was identified in studies of yeast COX-deficient *pet* mutants previously assigned to complementation group G22. To determine its subcellular location, Cox16p was tagged with a Myc epitope at the carboxy-terminus. This fusion protein complements the null mutant and is detected solely as an integral component of the mitochondrial inner membrane. Cox16p homologues are found in both the human and murine genomes, although human COX16 does not complement the yeast mutant. Cox16p does not appear to be involved in maturation of subunit 2, copper recruitment, or heme A biosynthesis. Cox16p is thus a new protein in the growing family of eukaryotic COX assembly factors for which there are as yet no specific functions known. *COX16* is a candidate for screening in inherited human COX deficiencies.

Acknowledgements

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Table of Contents

Acknowledgements.....

Abstract.....

List of Tables.....

List of Figures.....

List of Abbreviations.....

Chapter 1. Introduction.....1

Structure and Function of Cytochrome Oxidase.....2

Synthesis and Assembly of the Mitochondrially-Encoded COX Subunits.....8

Heme Biogenesis.....9

Generating the Cu_A and Cu_B Sites.....16

Other Assembly Factors.....22

Mammalian COX Assembly.....23

Human COX Assembly Factors.....25

Human Mitochondrial Disease.....30

Primary COX deficiency caused by mtDNA mutations.....31

Primary COX deficiency caused by nuclear mutations.....34

Secondary COX deficiency caused by nuclear mutations.....34

Summary.....38

Chapter 2. Materials and Methods.....40

Strains and Media: Bacterial and Yeast.....41

DNA Clones and Constructs.....42

Molecular Biology Techniques.....43

Biochemical Techniques.....49

Chapter 3. Results.....58

cox16 Mutants Are Defective in Cytochrome Oxidase Assembly.....59

Characterization of Cox16p.....69

Cox16p Has Mammalian Homologs.....75

Towards Identifying a Function for Cox16p.....78

Chapter 4. Discussion.....82

On the Characterization of Cox16p.....83

Bibliography.....93

List of Tables

Table 1.1. Relationship between subunits in bovine COX and yeast COX.....6

Table 2.1. Primer pairs used in the site-directed mutagenesis of yeast *COX16*.....46

Table 2.2. Primary antibodies used in this study.....55

Table 3.1. Metal Salt Suppression Analyses of a *cox*16 Null Mutant.....66

Table 3.2. Respiratory growth of *cox16* point-mutant strains.....81

List of Figures

Figure 1.1. Cytochrome oxidase mechanism of action.....3

Figure 1.2. Arrangement of COX subunits.....5

Figure 1.3. Transformation of Heme B to Heme A.....10

Figure 1.4. Heme A biosynthetic pathway.....14

Figure 1.5. Sco1p/Sco2p Chimeras.....19

Figure 1.6. Schematic representation of the assembly of COX.....24

Figure 3.1. Spectral analysis of mitochondrial cytochromes.....60

Figure 3.2. Western blot analysis of COX subunits.....62

Figure 3.3. Turnover of *in vivo* labeled mitochondrial translation products in wild type and *cox16* mutants.....63

Figure 3.4. Analysis of mitochondrial hemes in wild-type and mutant mitochondria.....65

Figure 3.5. The *COX16* gene encodes a putative membrane protein.....67

Figure 3.6. The *COX16* gene encodes a small, acidic protein.....68

Figure 3.7. Spectral analysis of aW303ΔCOX16/Myc1 mitochondrial cytochromes....70

Figure 3.8. Cox16p localizes to mitochondria.....71

Figure 3.9. Cox16p is an integral membrane protein.....73

Figure 3.10. Cox16p is an IMM protein.....74

Figure 3.11. Cox16-Myc is found in a higher molecular weight complex.....76

Figure 3.12. Cox16p has murine and human homologues.....77

Figure 3.13. hCOX16 does not complement a Sc*cox16* null mutant.....79

Figure 3.14. The amino acid sequence of Cox16p showing mutations introduced by site directed mutagenesis.....80

Figure 4.1. Schematic diagram of Cox16p orientation in the IMM.....88

List of Abbreviations

ADP	adenosine diphosphate
AMP	ampicillin
ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pair
cDNA	complementary DNA
COX	cytochrome oxidase
Co-IP	co-immunoprecipitation
Cu	copper
dpc	days post-coitum
EG	ethanol/glycerol
EST	expressed sequence tag
ETC	electron transport chain
FAD	flavin adenine dinucleotide
Gal	galactose
Hb	hemoglobin
HPLC	high pressure liquid chromatography
IMM	inner mitochondrial membrane
IMS	intermembrane space
kDa	kilodalton
LB	Luria-Bertani
LDH	lactate dehydrogenase

LS	Leigh Syndrome
LSFC	Leigh Syndrome, French Canadian
mtDNA	mitochondrial DNA
mRNA	messenger RNA
NADH	nicotinamide adenine dinucleotide
O.D.	optical density
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PMS	post mitochondrial supernatant
PMSF	phenylmethyl sulfonyl fluoride
rpm	rotations per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
TX-100	Triton X-100
WO	minimal glucose media
YPD	yeast peptone dextrose or rich glucose medium

Chapter 1: Introduction

Structure and Function of Cytochrome Oxidase

Mitochondria (*mitos* [thread] and *chrondros* [granule]) are present in the cytoplasm of all higher eukaryotes and are double membrane-bound organelles essential for the survival of aerobic cells, as they play a central role in cellular energy homeostasis. One of the main functions of mitochondria is the synthesis of adenosine triphosphate (ATP), an energy unit used for metabolic processes within cells. ATP synthesis occurs by the process of oxidative phosphorylation (OXPHOS) at the inner mitochondrial membrane (IMM) via the electron transport chain (ETC) (Michel et al. 1998). The ETC consists of four membrane-bound respiratory complexes (I/NADH dehydrogenase, II/Succinate dehydrogenase, III/cytochrome bc_1 complex, IV/cytochrome *c* oxidase), one peripheral protein (cytochrome *c*), and a hydrophobic molecule, Coenzyme Q (Capaldi 1990). These complexes transfer electrons in a specific series of reactions to complex IV, where the electrons are transferred to molecular oxygen (Figure 1.1). These electrons are generated from nicotinamide adenine dinucleotide (NADH, reduced form) and flavin adenine dinucleotide ($FADH_2$, reduced form), which are produced by oxidation of nutrients in the mitochondrial matrix via the citric acid cycle (Michel et al. 1998). Concomitantly, ETC complexes I, III and IV move protons out across the IMM, generating an electrochemical gradient across the membrane. The electrochemical gradient drives the synthesis of ATP by Complex V (also known as ATP synthase). Some of this ATP is used by the mitochondria, but most of it is exchanged for adenosine diphosphate (ADP), by the adenine nucleotide transporter (Smeitink et al. 2001). Complex IV of the ETC, or cytochrome *c* oxidase (COX), is a heterooligomeric complex, which functions as a dimer within the IMM (Tsukihara et al. 1996). COX has two main

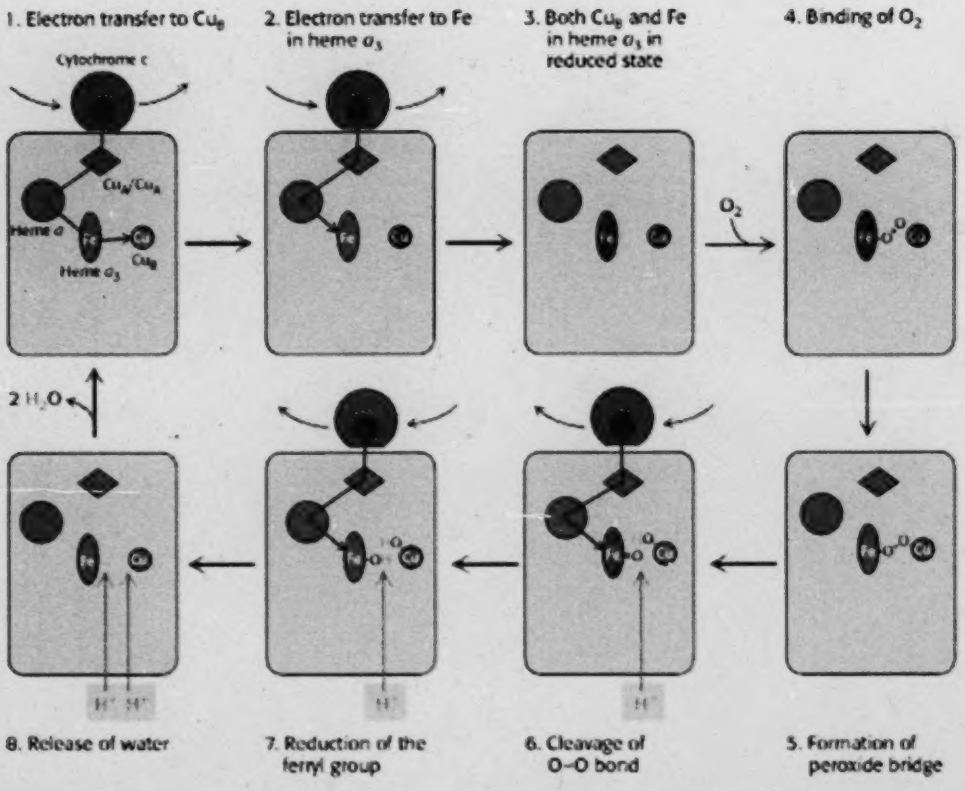

Figure 1.1. Cytochrome oxidase mechanism of action. The cycle begins with all prosthetic groups in their oxidized forms. Reduced cytochrome *c* introduces an electron that reduces Cu_B. A second reduced cytochrome *c* then reduces the iron in heme a_3. This Fe^{2+} center then binds oxygen. Two electrons are transferred to the bound oxygen to form peroxide, which bridges between the iron and Cu_B. The introduction of an additional electron by a third molecule of reduced cytochrome *c* cleaves the O-O bond and results in the uptake of a proton from the matrix. The introduction of a final electron and three more protons generates two molecules of H_2O, which are released from the enzyme to regenerate the initial state. The four protons found in the water molecules come from the matrix. Figure taken from Berg 2002.

functions in the ETC: (1) proton pumping, which contributes to the electrochemical gradient utilized by ATP synthase, and (2) catalyzing the transfer of electrons to molecular oxygen. COX is a member of the superfamily of heme- and copper-containing terminal oxidases that are found in both eukaryotes and prokaryotes (Michel et al. 1998). X-ray crystallography has been utilized to determine the structure of COX from beef heart (Tsukihara et al. 1995; Tsukihara et al. 1996; Tsukihara et al. 1996; Tsukihara and Yoshikawa 1998) and from the prokaryote, *Paracoccus denitrificans* (Iwata et al. 1995). These structures have yielded a detailed view of the architecture of the prosthetic groups and protein subunits. Mammalian COX, as determined by the bovine crystal structure (Tsukihara et al. 1995; Tsukihara et al. 1996; Tsukihara et al. 1996; Tsukihara and Yoshikawa 1998), is a 204 kDa, multimeric complex composed of 13 polypeptides (Figure 1.2, Table 1.1). Subunits I, II, and III are encoded in the mitochondrial genome and form the catalytic core of COX by virtue of their associated prosthetic groups (Capaldi 1990). Subunit I contains heme a and the heme a_3/Cu_B site, while subunit II contains the binuclear Cu_A redox center. The remaining 10 nuclear-encoded subunits are translated on cytosolic ribosomes and then imported into the mitochondrial matrix via the translocases of the outer and inner membrane (TOM and TIM complexes) (Stuart 2002). These nuclear-encoded subunits do not have homologs in bacteria (Iwata et al. 1995) and are thought to provide conformational stability to the catalytic core (Yoshikawa et al. 1998), or have a potential role in regulation of the COX holoenzyme during or after assembly, given that tissue specific isoforms exist in mammals for some of the subunits (IV, VIa, VIb, VIIa and VIII) (Poyton and McEwen 1996; Michel et al. 1998; Huttemann et al. 2000; Huttemann et al. 2001; Huttemann et al. 2003). Yeast *cox4*, *cox5a*, *cox5b*,

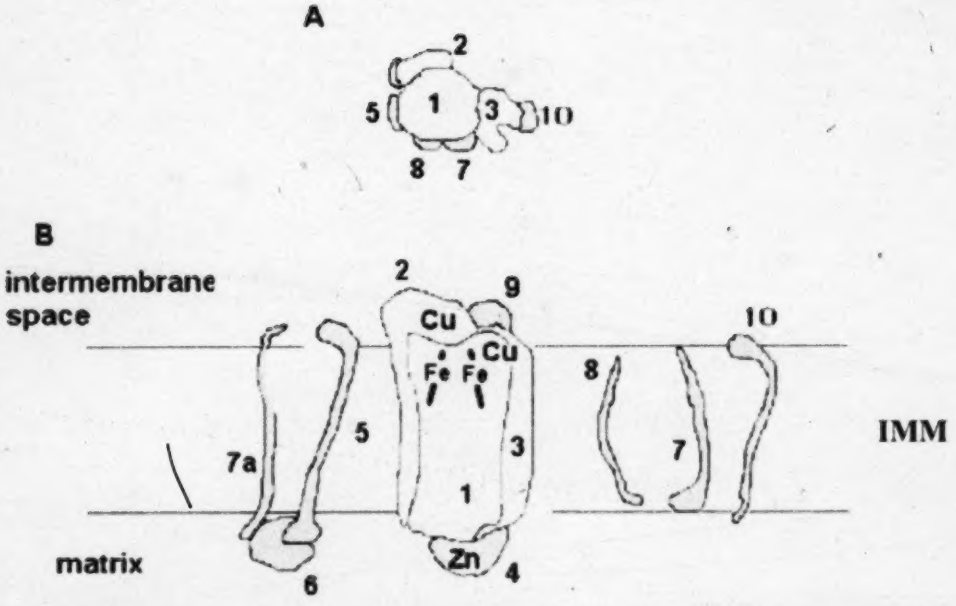

Figure 1.2. Arrangement of COX subunits. The core subunits are depicted in white and the eight cytoplasmically synthesized subunits are in gray. In the frontal view (B), some of the subunits have been drawn separately to show the core structure. Their relationship to the core is more evident in the diagram showing the enzyme viewed from the top (A). Figure from Barrientos et al. 2002.

Bovine	Yeast
I	1
II	2
III	3
IV	5a
Va	6
Vb	4
VIb	9
VIa	6a
VIc	7a
VIIa	7
VIIb	8
VIIc	
VIII	

Table 1.1. Relationship between subunits in bovine COX and yeast COX.

cox6, *cox7* and *cox9* deletion mutants lead to respiratory deficiency on non-fermentable carbon sources and are therefore essential for COX assembly and function (Poyton and McEwen 1996). The remaining yeast genes, *COX6a*, *COX7a*, and *COX8* are not necessary for the assembly of COX and have instead been demonstrated to encode subunit proteins shown to modulate COX enzymatic activity (Patterson and Poyton 1986; LaMarche et al. 1992; Taanman and Capaldi 1993). COX is also known to contain a zinc ion (bound to subunit Vb) (Tsukihara et al. 1996; Mochizuki et al. 1999), a magnesium ion (non-redox center present at subunit I and II interface), a sodium ion, and some phospholipids (Mochizuki et al. 1999). The roles of these ions and phospholipids are not known, but they may be required for stabilization of the COX complex (Tsukihara et al. 1996; Tsukihara and Yoshikawa 1998).

The biogenesis of COX is a complex process requiring the coordinated interaction of gene products from two separate genomes, the mitochondrial and the nuclear. Assembly of COX requires not only the polypeptides that form the complex itself, but a host of ancillary proteins, some of which are required to synthesize the prosthetic groups. As we develop a rudimentary understanding of all the processes involved in generating a functional COX enzyme, it is clear that a mutation in any requisite COX assembly factor results in a specific COX deficiency. COX biogenesis has been most widely studied in the yeast *Saccharomyces cerevisiae*, primarily through the screening of *petite* mutants. These mutants arise from mutations in nuclear genes and result in respiratory deficiency on non-fermentable carbon sources, while retaining the ability to grow on fermentable carbon sources such as glucose (Tzagoloff and Dieckmann 1990). The term *petite* comes from the observation that these cells form small colonies when grown on media

containing non-fermentable carbon sources with minimal glucose. COX assembly mutants generally share a characteristic biochemical phenotype that includes the absence of an absorption maximum at 603 nm, corresponding to heme aa_3, reduced steady state concentrations of Cox1p, Cox2p and Cox3p, as well as the *petite* phenotype (Barrientos et al. 2002). The yeast COX protein composition appears similar to the mammalian enzyme (Table 1.1), with the three largest protein subunits encoded by the mitochondrial DNA, while the nine remaining polypeptides are nuclear-encoded structural subunits (Taanman and Capaldi 1992). Based on genetic studies in yeast, it is now known that at least 30 nuclear-encoded proteins, which are not part of the final assembled COX complex, are involved in the COX assembly process (McEwen et al. 1986; Tzagoloff and Dieckmann 1990). It is clear from several studies that 30 is a minimum number and more proteins are likely involved in the assembly pathway. Nuclear-encoded COX assembly proteins can be currently classified into several categories: (1) those involved in synthesis and assembly of mitochondrially-encoded subunits, (2) those involved in heme synthesis, (3) those involved in copper transport and insertion into the COX holoenzyme, and (4) those that assist with assembly of the holoenzyme, but do not yet have any specific identified function. Delineation of these assembly pathways is essential, as it is the inability to complete proper assembly of COX that is the cause of most known human COX deficiencies.

Synthesis and Assembly of the Mitochondrially-Encoded COX Subunits

Numerous yeast nuclear-encoded proteins are essential for the synthesis and assembly of Cox1p, Cox2p and Cox3p into COX (Poyton and McEwen 1996). *COX1* is

known to require factors to assist in mRNA splicing (*MSS18*, *PET54*) (Seraphin et al. 1988; Valencik and McEwen 1991), RNA stability (*PET309*) (Manthey and McEwen 1995) and mRNA translation (*MSS51*, *PET309*) (Manthey and McEwen 1995; Perez-Martinez et al. 2003). *COX2* requires specific gene products for mRNA translation (*PET111*) (McMullin and Fox 1993; Mulero and Fox 1993), and export (*COX18*, *MSS2*) (Simon et al. 1995; Broadley et al. 2001; Saracco and Fox 2002) and maturation (*COX20*) of Cox2p (Hell et al. 2000). *COX3* requires three genes for mRNA processing to form a functional protein (*PET494*, *PET122*, *PET54*) (Valencik et al. 1989; McMullin and Fox 1993).

Current evidence suggests that Cox1p, Cox2p and Cox3p are inserted into the IMM by Oxa1p as they undergo translation on mitochondrial ribosomes (Hell et al. 2001; Stuart 2002). Failure of any of the Cox1p, Cox2p or Cox3p processing steps or insertion into the IMM results in failure of COX assembly and loss of respiratory competence (Poyton and McEwen 1996; Stuart 2002). Proper synthesis and assembly of Cox1p and Cox2p are necessary for insertion of their associated heme and copper prosthetic groups.

Heme Biogenesis

Heme A is an essential prosthetic group for the assembly and function of all eukaryotic, and some prokaryotic, cytochrome oxidases (Nobrega et al. 1990). Despite the importance of heme A, our knowledge of its anabolism from protoheme remains incomplete. The proposed pathway for heme A metabolism is shown in Figure 1.3. As heme is not covalently bound to the oxidase (Granick and Beale 1978), it is possible to extract heme from membranes using acidified acetone. Reverse-phase high pressure

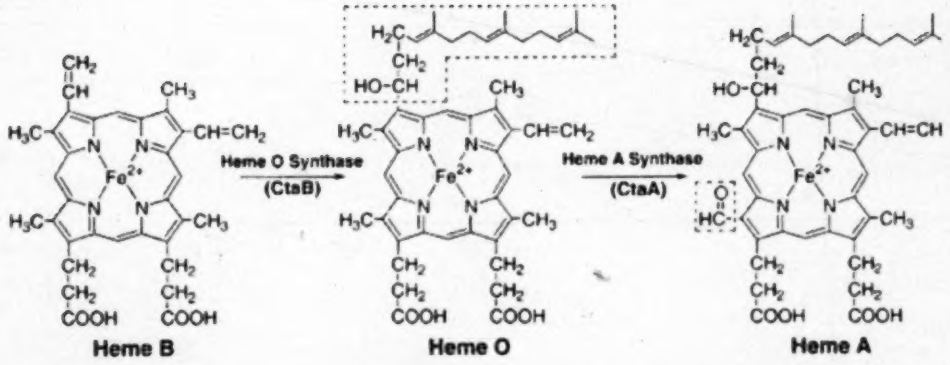

Figure 1.3. Transformation of Heme B to Heme A. Catalyzed by the enzymes Heme O Synthase (CtaB/CyoE/Cox10p/COX10) and Heme A Synthase (CtaA/Cox15p/COX15). Figure modified from Brown et al. 2002.

liquid chromatography (HPLC) allows separation of distinct types of heme and these separated peaks can then be purified and identified using various methods. Heme B (protoheme) is converted to heme O by exchanging a vinyl group on carbon two of the porphyrin ring with a hydroxyethyl farnesyl group (Puustinen and Wikstrom 1991). This step is carried out by heme farnesyl transferase (heme O synthase) and was first identified in *Escherichia coli* by demonstrating that the enzyme CyoE could catalyze the formation of heme O from heme B and farnesyl pyrophosphate (Puustinen and Wikstrom 1991; Saiki et al. 1992). Based on homology to CyoE in *E. coli*, subsequent studies in the prokaryote, *Bacillus subtilis*, and the eukaryote, *S. cerevisiae*, identified the proteins CtaB (Svensson et al. 1993) and Cox10p (Tzagoloff et al. 1993), respectively, to have heme O synthase activity. Mutations in either CtaB or Cox10p result in loss of both heme O and A from heme extractions of the bacterial membrane (CtaB) and yeast mitochondria (Cox10p) and thus suggest that heme O is the precursor of heme A.

The first heme A synthase was identified in *B. subtilis* (Svensson et al. 1993). Svensson et al (Svensson et al. 1993) demonstrated this heme A synthase activity by expressing plasmids containing CtaA and CtaB transformed into *E. coli*, which normally does not contain heme A. Therefore any heme A production would be due to CtaA and CtaB enzymatic activity (Svensson et al. 1993). Expression of CtaB alone resulted in an increase in heme O, but not heme A. In contrast, no increase in heme O, and very little heme A, was demonstrated with expression of CtaA alone, whereas co-expression of both CtaA and CtaB together resulted in much higher levels of both heme O and A. These results demonstrated that CtaA is the heme A synthase in *B. subtilis* and strengthened the evidence that heme O is a precursor of heme A. *B. subtilis* mutants defective in CtaA

were also found to lack heme A in their membrane extracts, while over-expression of CtaA resulted in an increase in heme A (Svensson and Hederstedt 1994). Heme A is thought to be formed from heme O by two successive monooxygenation reactions, which convert the methyl group on porphyrin ring D (on carbon 18) to a formyl group (Caughey et al. 1975; Brown et al. 2002). A monooxygenase is an enzyme that catalyzes the addition of a single oxygen atom to a substrate, with reduction of the other atom to water (Bernhardt 1995). This hypothesis is supported by experiments in the semi-anaerobic cyanobacterium, *Anacystis nidulans*, demonstrating that heme O and A are capable of transforming reversibly, depending on the environmental oxygen levels (Peschek et al. 1995). Additionally, in a reaction analogous to the heme O conversion to heme A, a methyl group on chlorophyll A is converted to a formyl group to generate chlorophyll B in plants (Porra et al. 1994). The only precursor required to complete this reaction is molecular oxygen. CtaA is thought to be a heme-dependent monooxygenase, based on findings that purified CtaA is found to bind both heme B and heme A (Svensson and Hederstedt 1994; Svensson et al. 1996) and the previously mentioned observation that the heme O to heme A conversion only requires molecular oxygen as a precursor.

Studies in *S. cerevisiae* have focused on Cox15p as the candidate monooxygenase, based on its weak sequence identity to CtaA. In addition to being COX-deficient, *cox15* null mutants contain low amounts of heme O and lack heme A, as judged by HPLC analyses of mitochondrial heme extractions (Barros et al. 2001). This phenotype contrasts with *cox10* null mutants, which lack both heme O and heme A. This strongly suggests a role for Cox15p in heme A synthesis. Cox15p may be a cytochrome P450-type monooxygenase, which would predict that it obtains electrons from a NADH-

dependent, FAD-containing reductase via an iron-sulfur protein of the 2Fe-2S type (Bernhardt 1995). Consistent with this suggestion, *COX15* in *Schizosaccharomyces pombe* has been found to be fused with *YAH1* (yeast adrenodoxin homolog 1) (Barros and Nobrega 1999), a mitochondrial adrenodoxin that functions in iron-sulfur cluster biogenesis and is, in fact, an iron-sulfur cluster containing protein (Lange et al. 2000). Over-expression of both Cox15p and Yah1p in *S. cerevisiae* results in increased levels of heme A, beyond that seen when expressing Cox15p alone (Barros et al. 2002). The third component of this pathway is believed to be *ARH1* (adrenodoxin reductase homolog 1) whose protein product, Arh1p, accepts electrons from NADH and transfers them to Yah1p. These results suggest that, similar to other mitochondrial P450 monooxygenases, Cox15p functions in heme A synthesis as part of a three component monooxygenase system composed of Arh1p (ferrodoxin reductase), Yah1p (ferrodoxin) and Cox15p. This pathway is summarized in Figure 1.4.

Regulation of the heme A biosynthesis pathway is beginning to be elucidated (Barros and Tzagoloff 2002). Studies have shown that Cox15p is a positive regulator of heme B farnesylation. Contrary to what would be expected, given that heme O is a precursor of heme A, loss of Cox15p results in decreased levels of heme O. The sedimentation rate of Cox15p is not different between wild type and *cox10* cells, suggesting that Cox10p and Cox15p function independently. This also suggests that some other intermediate, metabolite, or mechanism of Cox15p may be required for heme O synthesis (Barros and Tzagoloff 2002; Brown et al. 2002). However, it has been demonstrated by co-immunoprecipitation (Co-IP) experiments that CtaA and CtaB are found together in a complex (Brown et al. 2004). This observation either suggests that

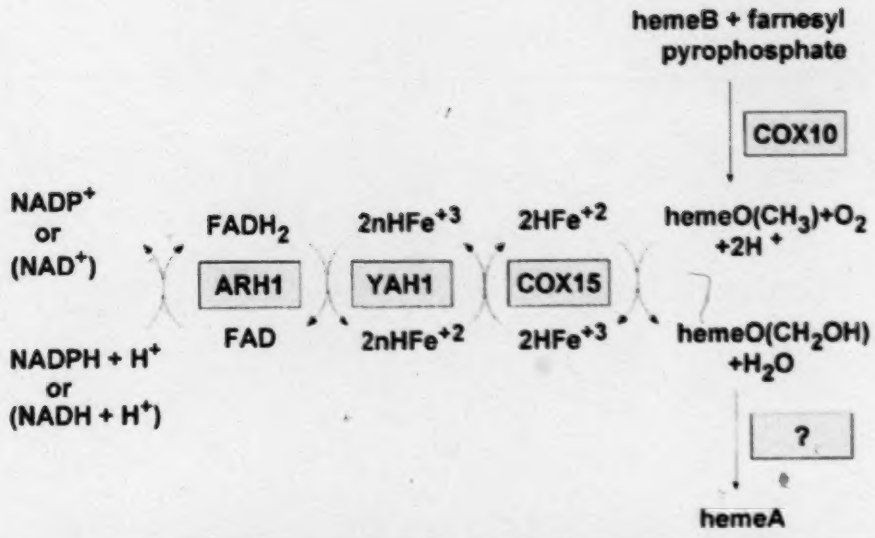

Figure 1.4. Heme A biosynthetic pathway. The conversion of heme B to heme O is catalyzed by Cox10p, a farnesyl transferase. Arh1p (adrenodoxin reductase), Yah1p (adrenodoxin), and Cox15p are proposed to catalyze the conversion of the methyl group at C8 of heme O, to heme A, by two successive monooxygenation reactions through, as yet, unidentified processes. nHFe refers to the non-heme iron of adrenodoxin and HFe to the heme iron of Cox15p. Figure taken from Barrientos et al. 2002.

Cox10p and Cox15p have a weak transient interaction not detected by previous methods, or that they have distinct or non-overlapping functions from CtaA and CtaB. Co-IP and crosslinking studies for Cox10p and Cox15p have not been reported. Based on the heme O and A profiles of various COX assembly mutants, it was also found that oxygenation or formylation of heme O to heme A is coupled to, and regulated by COX assembly, since mutants in any of several COX assembly factors fail to accumulate heme A (Barros and Tzagoloff 2002). It remains to be determined whether it is an assembled COX or COX function that is necessary for heme A synthesis. In addition, the mechanism of heme A insertion into Cox1p has not yet been characterized.

It has been demonstrated that heme A is not essential for assembly of COX in *Rhodobacter sphaeroides* (Hiser and Hosler 2001). Heme A may be inserted at multiple points during assembly of the holoenzyme and COX does not appear to require a linear assembly pathway (Hiser and Hosler 2001), as is the case in eukaryotes (Glerum et al. 1997). The significance of this finding is poorly understood, but does highlight that differences in assembly do exist between prokaryotes and eukaryotes, which may limit the usefulness of prokaryotic models in understanding of human COX assembly and deficiency. Much work remains to be done to delineate the heme A synthesis pathway and it is entirely probable that it is also subject to other as-yet unknown regulatory influences. Unfortunately, our understanding of heme A synthesis lags behind that of the acquisition of the other major prosthetic group, copper.

Generating the Cu_A and Cu_B Sites

The trace element copper is an essential prosthetic group for the function of COX (Glerum et al. 1996). COX contains three copper molecules, one of which is part of the heme a_3/Cu_B site of subunit I, while the other two molecules form the binuclear Cu_A site in subunit II (Tsukihara et al. 1995; Tsukihara and Yoshikawa 1998). Copper is essential due to its ability to adopt distinct redox states, either oxidized Cu(II) or reduced Cu(I) (Pena et al. 1999). This allows copper to act as an important catalyst in the transfer of electrons in COX. Although essential, copper is a highly reactive and cytotoxic ion when allowed to accumulate in excess of cellular needs (Pena et al. 1999; Field et al. 2002). As a result, cells have evolved homeostatic mechanisms to prevent free copper ions in the cell, thus preventing undesirable side-reactions from occuring (Field et al. 2002). This sequestration has forced cells to find specific ways of delivering copper to sites where it is needed, such as to the mitochondria, in the case of COX. Evolution has also solved this delivery problem through the use of low molecular weight proteins called copper chaperones (Field et al. 2002). These molecules are able to bind copper ions and deliver them safely in the cell. While three copper chaperone pathways have been shown to exist in eukaryotic cells (Winge et al. 1998; Field et al. 2002), for the purpose of this review, I will focus on copper delivery to the mitochondria for assembly of COX. As with other COX assembly pathways, much of the research into COX copper metabolism has utilized *S. cerevisiae* as a model organism. At least four yeast proteins, Cox11p (Hiser and Hosler 2001), Cox17p (Glerum et al. 1996), Sco1p and Sco2p (Glerum et al. 1996), are implicated in the transport and insertion of copper into COX. These proteins are conserved throughout many species, suggesting essential cellular functions (Amaravadi et

al. 1997; Punter et al. 2000). Cox17p has been proposed to have a role in shuttling Cu(I) to the mitochondria, whereas Cox11p and Sco1p are thought to be involved with copper insertion into Cox1p and Cox2p, respectively (Glerum et al. 1996; Glerum et al. 1996; Rentzsch et al. 1999; Hiser and Hosler 2001). Sco2p has also been proposed to have a role in copper metabolism, but its role has yet to be defined (Glerum et al. 1996).

The initial mitochondrial copper chaperone for COX is Cox17p (Glerum et al. 1996). Cox17p is a small, 69 amino acid hydrophilic protein found in two distinct cellular compartments, the cytoplasm (40%), and the mitochondrial intermembrane space (IMS)(60%) (Glerum et al. 1996; Beers et al. 1997). The mechanism by which this distribution occurs has not been reported in the literature. *cox17* null mutants exhibit a phenotype consistent with that of other COX assembly factor mutants: a failure of COX to properly assemble as determined by cytochrome aa_3 spectral loss, and respiratory deficiency on non-fermentable carbon sources (Glerum et al. 1996). They also demonstrate a marked decrease in Cox1p, Cox2p, and Cox3p while all other subunits are present at wild-type levels. The *cox17* null mutant is capable of being rescued by the addition of high concentrations of exogenous copper (0.4% copper) to the media, which in yeast appears to be unique to the *cox17* mutant phenotype. Loss of Cox17p does not appear to affect the function of cytosolic copper enzymes, suggesting its role is specific to mitochondria. Each Cox17p chaperone is thought to bind 2-4 atoms of copper (Beers et al. 1997; Srinivasan et al. 1998; Heaton et al. 2001; Palumaa et al. 2004), via the cysteine (Cys) residues of the KPCCXC motif (Amaravadi et al. 1997). Cox17 proteins that have one of these three Cys residues mutated to serine retain the ability to bind Cu(I) and localize to the IMS, but do not retain COX activity (Heaton et al. 2000).

Simultaneous mutations in two of these three Cys residues result in failure to bind Cu(I), but Cox17p still retains its localization to the IMS (Heaton et al. 2000). This observation suggests that localization of Cox17p is independent of copper binding. Cox17p is proposed to shuttle copper from the cytoplasm to the IMS, where it is thought to transfer copper to Sco1p (Glerum et al. 1996). This hypothesis is supported by the observations that purified Cox17p appears to transfer copper to Sco1 and Cox11p, as demonstrated by *in vitro* assays (Horng et al. 2004). However, Cox17p appears to be functional when tethered to the IMM (Maxfield et al. 2004). If this latter observation is correct, it would suggest that it is not essential to Cox17 function for the protein to cycle between the cytoplasm and the IMS, where it interacts with Cox11p and Sco1p.

Yeast *SCO1* and its homolog, *SCO2*, were first implicated in the COX copper pathway by their ability to rescue the *cox17* respiratory deficiency when present at high copy number (Glerum et al. 1996). Studies of chimeras have demonstrated that a 13 amino acid segment next to the transmembrane domain is critical for function of Sco1p and cannot be replaced by its Sco2p counterpart, which differs at only 3 amino acid positions (Figure 1.5) (Lode et al. 2002). The whole amino-terminus (amino acids 1-75) and most of the carboxy-terminus (amino acids 106-295) of Sco1p are capable of being replaced by the homologous regions of Sco2p without loss of function. *SCO2* was only capable of rescuing a *sco1* point mutant (not a *sco1* null mutant), in the presence of slightly elevated copper concentrations, suggesting a potential role in mitochondrial copper metabolism (Glerum et al. 1996). Loss of *SCO2* does not result in a respiratory deficient phenotype. Sco2p has also been demonstrated to interact with Cox2p, suggesting a potential functional role with the binuclear Cu_A site (Lode et al. 2002).

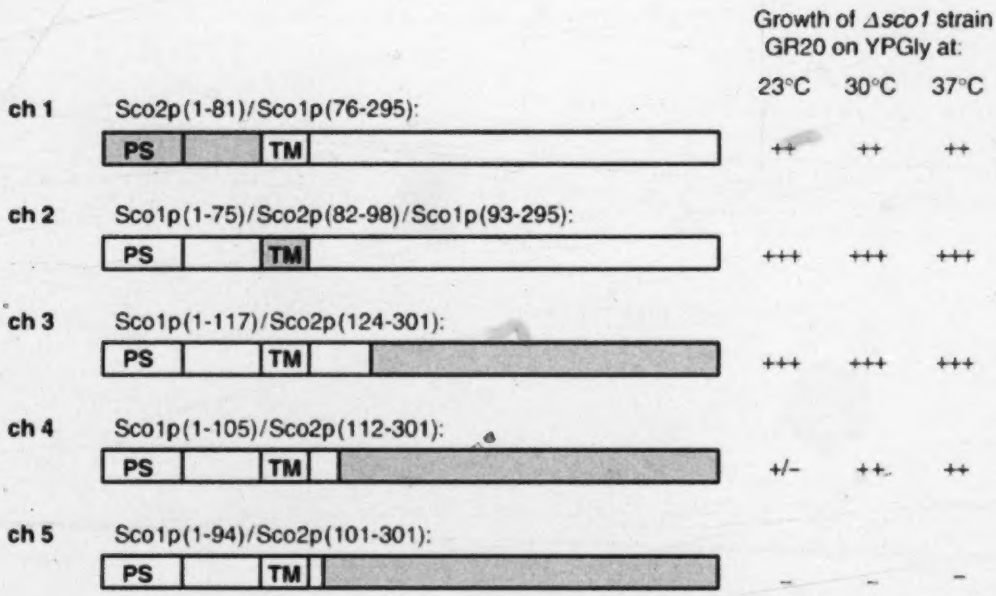

Figure 1.5. Sco1p/Sco2p chimeras: Sequences derived from Sco1p are shown in white, those from Sco2p in grey. PS indicates the mitchondrial presequence and TM the TM segment. Growth of Δ*sco1* strain GR20 transformed with the respective chimeric constructs was tested on non-fermentable glycerol medium: +++, wild-type growth; ++, reduced growth; +/-, barely detectable growth; -, no growth. Figure taken from Lode et al. 2002.

Although the Sco1p and Sco2p sequences are 50% identical, their null mutants have different phenotypes as described above, which suggests these two proteins have non-overlapping functions (Glerum et al. 1996).

Sco1p is a 30-kDa integral IMM protein (Buchwald et al. 1991). Mutation of *SCO1* results in the specific loss of Cox1p and Cox2p, subsequent COX misassembly, and a petite phenotype (Buchwald et al. 1991; Glerum et al. 1996). Sco1p contains the CXXXC motif, which binds one atom of copper per molecule of protein, with a higher affinity than Cox17p. This motif is similar to the amino acid sequence found in Cox2p (Nittis et al. 2001; Beers et al. 2002), which has also been shown to bind copper (Coruzzi and Tzagoloff 1979). The CXXXC motif of Sco1p is located in its carboxy-terminus, which is found within the IMS (Nittis et al. 2001), and is absolutely conserved between all mitochondrial Sco proteins and their bacterial homologs (Balatri et al. 2003). Mutation of the Cys residues in this motif results in loss of function of Sco1p (Rentzsch et al. 1999). The *sco1* null mutant is not rescued by the addition of exogenous copper or by over-expression of *COX17*, indicating that Sco1p functions downstream of Cox17p. Genetic studies have also demonstrated that proper localization and orientation in the IMM are essential for Sco1p function (Beers et al. 2002). Native Sco1p persists in a complex with a mass of approximately 88 kDa and is believed to exist as a homo-trimer that can form independently of copper ions (Beers et al. 2002; Lode et al. 2002). Two alternate models have been proposed for the function of Sco1p: (1) that it is involved in transferring Cu(I) directly from Cox17p to COX (Glerum et al. 1996), or (2) that based on sequence similarity of Sco1p/Sco2p to peroxiredoxins and thiol:disulfide oxidoreductases, Sco1p is required for reduction of the Cys residues comprising the

CXXXC copper-binding motif of Cox2p (Chinenov 2000; Balatri et al. 2003). The solution structure of a purified bacterial apo-Sco1 has been solved and suggests that Sco1 does have a thioredoxin-like fold, with weak similarity to peroxiredoxins, but these data do not give any further insight into Sco1p function (Balatri et al. 2003). While it is unclear if either of these models for the function of Sco1p is correct, Sco1p has been demonstrated to physically interact with Cox2p (Lode et al. 2000). As with the formation of the homo-trimer, this interaction is capable of occurring independently of copper. This observation, along with the finding that a S240F mutation in Sco1p results in a specific loss of Cox2p, suggests Sco1p specifically interacts with the Cu_A site at this amino acid (Dickinson et al. 2000; Lode et al. 2002; Punter and Glerum 2003). Another possibility exists that the S240F mutation may only affect the Sco1p interaction with the Cu_A site, leaving its interaction with the Cu_B site functional. If Sco1p only interacts with the Cu_A site, however, it also suggests the existence of another protein chaperone that must transfer copper to the Cu_B site on Cox1p (Dickinson et al. 2000). This hypothesis is supported by studies of the *Bacillus subtilis* SCO homolog, YpmQ, which have demonstrated YpmQ is required for assembly of the caa_3 oxidase, but not for an oxidase that only contains a Cu_B site (Mattatall et al. 2000).

Based on studies in *Rhodobacter sphaeroides*, Cox11 was prososed to be a Cu_B-specific chaperone (Hiser and Hosler 2001). Using electron paramagnetic resonance spectroscopy, it was demonstrated that loss of Cox11 results in a misassembled COX that is specifically missing its Cu_B site, but does contain hemes a and a_3, and the Cu_A site. This *cox11* mutant was also found to have a marked reduction of metal at the magnesium/manganese binding site between subunits I and II. Yeast Cox11p is a 34-kDa

integral protein of the IMM (Tzagoloff et al. 1990). Loss of *COX11* results in a *petite* phenotype when grown on a non-fermentable carbon source. The RNA processing and protein synthesis of Cox1p, Cox2p and Cox3p in *cox11* mutants were found to be comparable to wild-type, suggesting that Cox11p functioned post-translationally (Tzagoloff et al. 1990). Cox11p exists as a dimer and each of these polypeptides may be capable of binding a Cu (I) (Carr et al. 2002). Mutation of any of three conserved Cys residues (Cys-111, Cys-208, Cys-210) has been observed to decrease Cu (I) binding affinity, reduce COX activity and confer a *petite* phenotype.

Other Assembly Factors

As discussed in the previous sections, COX assembly factors are protein products that when deficient, exhibit a COX-specific *petite* phenotype. In contrast to the other COX assembly factors described thus far, the role of the following factors in COX assembly has not yet been elucidated: *COX16*, *COX19*, *COX23*, *PET117* and *PET191*. Cox19p and Cox23p are believed to have potential roles in metal transport (Nobrega et al. 2002; Barros et al. 2004). Cox19p has been demonstrated to have dual localization in the cytoplasm and IMS (Nobrega et al. 2002), similar to that reported for Cox17p (Glerum et al. 1996; Beers et al. 1997). This finding, along with the observation that Cox19p contains four Cys residues that align with those in Cox17p, suggests that Cox19p may have a role in metal transport (Nobrega et al. 2002). Cox23p is a soluble protein which, similarly to Cox17p and Cox19p, has dual localization in the cytosol and IMS. Cox23p is also proposed to have a role in the copper recruitment pathway. Null mutants of *cox23* are rescued by high concentrations of exogenous copper in the presence of over-

expressed *COX17* (Barros et al. 2004). However, neither Cox17p over-expression or exogenous copper individually are sufficient to rescue the respiratory deficiency of *cox23*. Functions for three other proteins - Cox16p, Pet117p, and Pet191p - remain to be elucidated. Cox16p is the focus of this thesis and its characterization is described in chapters 3 and 4 (Carlson et al. 2003).

Mammalian COX Assembly

As in *S. cerevisiae*, the process of COX biogenesis in humans has yet to be completely elucidated. Evidence from several studies has allowed a model for COX assembly in humans to be developed. Through the use of metabolic labeling, translational inhibitors, and two-dimensional gel electrophoresis, a series of four complexes (S1-S4) have been observed to form in a distinct structural and temporal assembly pathway (Figure 1.6). Assembly is thought to start with COX1 (S1) binding COX4, followed by heme A insertion to form S2 (COX5a may potentially be inserted into the complex also) (Taanman and Williams 2001). Experiments in rats have demonstrated that COX assembly is a slow process, with COX1 taking up to 1.5 hours to assemble with COX2 and COX3 (Wielburski and Nelson 1983). It has been proposed that this time interval is required to allow COX1 to be folded into the IMM and have the two heme A molecules inserted (Taanman and Williams 2001). Alternatively, it may be possible that synthesis of heme A is the rate-limiting step, as binding of heme A to COX1 appears to promote binding to subunits II and III (Wielburski and Nelson 1984). The answer to this question remains to be elucidated. Subcomplex S3 is missing only the polypeptides COXVIa, COXVIIa and COXVIIb. Based on the bovine crystal structure,

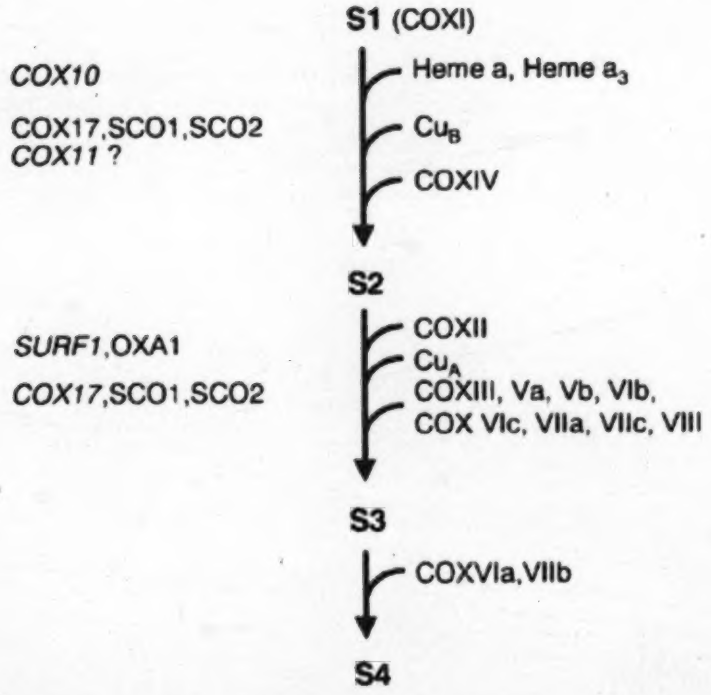

Figure 1.6. Schematic representation of the assembly of COX. S1-S3 refer to the three subcomplexes and S4 to the holoenzyme complex. The addition of prosthetic groups and structural subunits is indicated on the right side of the figure and the genes thought to be important at different stages of assembly are shown on the left. Human homologues of several other yeast COX assembly factors have been reported, but their role in the assembly process remains unknown. Figure from Nijtmans et al. 1998.

these three proteins are found on the periphery of COX and would be expected to assemble last (Tsukihara et al. 1996). Subunit COXVIa is not required for assembly of COX in yeast (Cox6a), but rather modulates COX enzymatic activity (Taanman and Capaldi 1993). This suggests that COX VIIa and/or COXVIIb are required to complete formation of the COX holoenzyme (S4) and subsequent dimerization in the IMM. While only a few subcomplexes have been identified, this does not exclude the possibility for others, as S2 and S3 may simply represent rate-limiting steps with more remaining to be identified (Nijtmans et al. 1998). Early studies in yeast have not identified any similar subcomplexes (Glerum and Tzagoloff 1998), however, these experiments were performed in the context of COX null mutants. The lack of intermediates may be due to COX misassembly and degradation of the various COX subunits by the proteolytic machinery (Rep and Grivell 1996).

Human COX Assembly Factors

Several methods have been utilized to identify human COX assembly genes, including functional complementation of yeast mutants, homology searches of expressed sequence tag (EST) databases, and microcell-mediated chromosome transfer. Human *COX10* was first identified by functional complementation of a yeast *cox10* null mutant with a high-copy plasmid (Glerum and Tzagoloff 1994). Over-expression of COX10 may be necessary due to the heterologous yeast environment that causes the COX10 to be unstable or partially inactive when expressed in yeast. *COX10* is found on chromosome 17p and consists of 7 exons (Murakami et al. 1997). *COX10* lacks both a CAAT and TATA-promoter element and is ubiquitously expressed in all tissues, suggesting that it

may be a housekeeping gene. The S2 subcomplex (Figure 1.6) is not detected in fibroblast cells that are *COX10* deficient, which suggests that heme A incorporation is required before COX1 can bind COX4 and COX5a (Williams et al. 2004).

COX15 was first identified by screening the human EST database, using the yeast Cox15p sequence as a query (Petruzzella et al. 1998). *COX15* localizes to chromosome 10q24 and is ubiquitously expressed in all tissues. *In vitro* mitochondrial import assays have demonstrated that human COX15 is imported into mitochondria.

As with the heme A synthetic pathway, human homologs and paralogs have been identified for all the known yeast genes involved in the COX copper pathway. This suggests the copper recruitment pathway between yeast and humans is at least partly conserved. Human *COX17* was first identified by its ability to rescue a yeast *cox17* null mutant (Amaravadi et al. 1997). *COX17* localizes to the long arm of chromosome 3 and is ubiquitously expressed in all tissues (Punter et al. 2000). In addition to human *COX17*, homologs have been identified in mouse (Takahashi et al. 2001), pig (Chen 1997), and rat (Kako et al. 2000). All three mammalian proteins contain the copper-binding KXCCXC motif (Amaravadi et al. 1997). Studies in mouse and rat have found *COX17* mRNA to be ubiquitously expressed, similar to what is observed in humans (Kako et al. 2000; Takahashi et al. 2001). *cox17* deficient mice (*COX17* knockout) have been generated (Takahashi et al. 2002). Heterozygous mice had a 20% decrease in COX activity compared with wild-type, but were otherwise healthy. Homozygous mutant embryos die between E8.5-E10 dpc and demonstrate a severe deficiency in COX activity in both embryonic and extraembryonic tissues. The homozygous *cox17* null mouse phenotype

demonstrates how essential COX activity is for survival of mammals and highlights the need for more animal models to characterize human COX deficiency.

Human *SCO1* (Petruzzella et al. 1998) and *SCO2* (Papadopoulou et al. 1999) genes have also been identified. While highly homologous to the yeast *SCO1*, h*SCO1*, and h*SCO2* are unable to functionally complement a yeast *sco1* null mutant (Paret et al. 1999). Genetic analysis of the h*SCO1* and h*SCO2* genes suggests they arose from independent gene duplications and are paralogs, rather than homologs, of yeast *SCO1* (Papadopoulou et al. 1999). Human SCO1 and SCO2 appear to have independent functions in Cu(I) delivery to COX (Leary et al. 2004). Over-expression of COX17 rescues a COX deficiency in *sco2*, but not *sco1*, patient cell lines. Size exclusion chromatography suggests that SCO1 and SCO2 both exist as homo-dimers.

Human *SCO1* is found on chromosome 17p12-p13 and is ubiquitously expressed in all tissues (Petruzzella et al. 1998). *In vitro* import assays (Petruzzella et al. 1998) and fluorescence microscopy (Paret et al. 1999) demonstrate that SCO1 localizes to the mitochondria. *sco1* deficient fibroblast cells have been observed to accumulate the S2 subcomplex (Figure 1.6). This may be due to COX2 only associating with the S2 subcomplex after formation of the Cu_A center (Williams et al. 2004). Therefore, if the Cu_A site does not form, S2 is not further assembled. Human *SCO2* localizes to chromosome 22q13 and is ubiquitously expressed in all tissues, with highest expression in brain, heart, skeletal muscle, liver and kidney (Papadopoulou et al. 1999). SCO2 contains the copper-binding motif CXXXC and has been demonstrated to bind copper in a 1:1 protein/copper atom ratio (Jaksch et al. 2001). It has also been observed in fibroblasts, myoblasts and myotubes that *sco2* mutations causing COX deficiency can be

rescued by the addition of $CuCl_2$ to the growth media (Salviati et al. 2002). These two findings strongly suggest a role for SCO2 in the copper acquisition pathway. Although SCO2 has a weak similarity to thiol: disulfide oxidoreductases, it is incapable of reducing disulfide bridges, making it less likely that the function of SCO2 is to reduce the Cys residues of the Cu_A site in COX2 (Jaksch et al. 2001). Two pathogenic SCO2 mutations, E140K (E155K in yeast) and S225F (S240F in yeast), from patients who died of fatal infantile cardioencephalopathy with COX deficiency, were tested in yeast (Dickinson et al. 2000). The E155K mutant was not observed to have an abnormal phenotype, while the S240F mutant results in a misassembled COX complex with a specific absence of Cox2p (Dickinson et al. 2000). This suggests that the main role of human SCO2 is copper transfer to the Cu_A site of COX2.

Similar to the other proteins known to function in the copper pathway, Cox11p has also been shown to have a human homolog. Human *COX11* localizes to chromosome 17q22 and is ubiquitously expressed in all tissues (Petruzzella et al. 1998). *In vitro* import assays have demonstrated that COX11 is imported into the mitochondria and is believed to exist as a homo-dimer *in vivo* (Leary et al. 2004).

Of all COX assembly factors known to be involved in human disease, *SURF1* is unique in that the human gene was identified before its yeast homolog, *SHY1* (Surf Homolog of Yeast One) or before its known association to disease (Mashkevich et al. 1997). *shy1* null mutants appear to be blocked before COX2 incorporation (Tiranti et al. 1999). Yeast *shy1* mutants show a similar biochemical phenotype to *surf1* in that they only produce 10-15% of a fully assembled, and apparently functional, COX (Barrientos et al. 2002). These mutants show a pleiotropic phenotype including inefficient electron

transport and a 2-3 fold increase of succinate- and NADH cytochrome *c* reductase activity, in addition to decreased COX activity. These mutants have a *petite* phenotype despite 20% COX activity (Mashkevich et al. 1997).

SURF1 localizes to chromosome 9q34 (Yon et al. 1993), in a cluster of conserved housekeeping genes called the surfeit locus (Yao and Shoubridge 1999). This locus is highly conserved in eukaryotes and prokaryotes, suggesting that SURF1 function may be essential (Pecina et al. 2004). *SURF1* has a TATA-less promoter (Cole and Gaston 1997) and is ubiquitously expressed in all tissues, with slightly lower mRNA signal in brain tissue (Yao and Shoubridge 1999). *SURF1* encodes a 35-kDa protein that is processed to 30-kDa in the mitochondria (Yao and Shoubridge 1999) and has been observed to persist in a 250-kDa complex of unknown composition (Nijtmans et al. 2001). SURF1, similar to yeast Shy1p, contains a typical amino-terminal mitochondrial targeting sequence and has been demonstrated to localize to the IMM (Mashkevich et al. 1997; Yao and Shoubridge 1999). Human *surf1* cell lines do not contain *SURF1* mRNA transcripts and are not rescued by *SHY1*, even though Shy1p correctly localizes to the mitochondria (Tiranti et al. 1999). The absence of functional complementation is thought to either reflect slightly different functions for SURF1 and Shy1p, or altered function/stability of the Shy1p in the heterologous human environment (Tiranti et al. 1999). No difference in the mitochondrial DNA (mtDNA)-specific translation products are observed between *surf1* mutants and controls suggesting that a role for SURF1 in mtDNA processing is unlikely (Tiranti et al. 1999). *shy1* mutants have been found to be rescued by over-expression *MSS51* (Barrientos et al. 2002), which is involved in translation of COX1 mRNA (Perez-Martinez et al. 2003). At the molecular level, *surf1* null cells have 30%

COX activity and, as demonstrated by blue native polyacrylamide gel electrophoresis (BN-PAGE), only 20-30% of the COX enzyme is present when compared to wild-type cell COX levels (Coenen et al. 1999). This is consistent with the observation that steady state levels of both nuclear- and mitochondrially-encoded COX subunits are ubiquitously reduced in patients' cells (Yao and Shoubridge 1999). Further BN-PAGE demonstrated the accumulation of COX subcomplexes containing COXI alone (S1) or COXI, COXIV and COXVa (S2) (Figure 1.6) (Tiranti et al. 1999).

A constitutive knockout of mouse *Surf1* has been developed (Agostino et al. 2003). Embryonic lethality was observed starting at E4 dpc, in over 90% of the *Surf1*$^{-/-}$ mice. Histologically, the surviving mice demonstrated severe morphological abnormalities in skeletal muscle. They were also observed to be severely impaired in muscle strength. These mice were also found to have a generalized COX deficiency in liver and, to a much lesser extent, in heart and brain. In contrast to human *surf1* deficiency, the *Surf1*$^{-/-}$ mice do not exhibit abnormal brain morphology or neurological symptoms. However, similar to the human disease (Leigh Syndrome, as will be described), there was substantial phenotypic variability between mice with identical *Surf1* alleles. This phenotypic heterogeneity may be due to differences in nuclear background and potential epigenetic factors.

Human Mitochondrial Disease

Given its many structural subunits, the numerous assembly factors involved, and its requirement for components encoded by genes in two distinct genomes, it is not surprising that errors can arise in the assembly of the COX holoenzyme. As

mitochondria are the major source of cellular energy, it is also not surprising that mutations in proteins resulting in COX deficiencies cause a wide range of phenotypes, but tend to affect those tissues with the highest energy demands, such as the neuromuscular and endocrine systems. While most of our knowledge of OXPHOS diseases comes from studies of the mitochondrial genome, the last two decades have seen an explosion of research on nuclear-encoded COX genes and resulted in the characterization of numerous COX deficiencies. Analyses of these COX deficiencies has resulted in a dual classification of: (1) primary COX deficiency, which is caused by mutations in mtDNA or nuclear genes that encode structural subunits, and (2) secondary COX deficiency that is caused by mutations in genes that affect COX assembly or conformational stability, but whose gene products are not part of the final assembled COX complex (Yoshikawa et al. 1998).

Primary COX deficiency caused by mtDNA mutations

Since 1981, when the mtDNA sequence was first published (Anderson et al. 1981), though the discovery of the first pathogenic mtDNA mutations in 1988 (Holt et al. 1988; Wallace et al. 1988; Wallace et al. 1988), we have seen rapid advances in the field of mitochondrial genetics. We have gone from one known point mutation to well over 150 point mutations and mtDNA rearrangements that have been described (DiMauro and Schon 2001). The human mitochondrial genome is a small, intronless, circular DNA molecule that encodes 37 genes (Anderson et al. 1981; Taanman 1999). Of these, two code for ribosomal RNAs, 22 for transfer RNAs that are essential for the translation of

mtDNA, and 13 genes that encode structural subunits of the ETC, with 3 of these (COX I, II, and III) being specific for COX.

Mitochondrial genetics are different from classical Mendelian genetics in several ways (DiMauro and Schon 2001): (1) mtDNA is maternally inherited; (2) polyplasmy – while most human cells contain 2 copies of each nuclear gene, each mitochondrion has many copies of its genome, resulting in hundreds to tens of thousands of mitochondrial genomes per cell; (3) heteroplasmy - patients with mtDNA mutations often exhibit heteroplasmy, whereby a combination of mutant and wild type mitochondrial genomes may be present in a cell. The percentage of mutant DNA can vary widely between individuals and between tissues, or even cells, within the same patient (Chinnery and Schon 2003). A threshold effect has also been demonstrated to exist, whereby a certain percentage of mutant mitochondria are required before the development of any symptoms of disease; (4) mitotic segregation - when cells divide, the proportion of mutant mtDNA in daughter cells can shift. Thus the level of mutant mtDNA can change during transmission from a mother to her child, likely due to a bottleneck effect at oogenesis. This bottleneck effect is essentially a random sampling of mtDNA from the mother that can result in drastic changes in the percentage of mutant mtDNA in the child.

In the same way that these features make mtDNA genetics distinct, they also result in highly variable clinical features associated with mtDNA mutations (Dahl and Thorburn 2001; Shoubridge 2001). 11 mutations have now been described in the three mitochondrially-encoded subunits of COX, presenting with a broad range of clinical phenotypes (Shoubridge 2001). Four mutations have been described for *COX1*: (1) a heteroplasmic nonsense mutation resulting in a multisystem disorder (Bruno et al. 1999);

(2) a heteroplasmic nonsense mutation resulting in a pure myopathy and recurrent myoglobinuria (Karadimas et al. 2000); (3) a heteroplasmic 5 bp microdeletion in the 5' end of *COX1* associated with motor neuron disease (Comi et al. 1998); (4) missense mutations resulting in mitochondrial iron overload and acquired idiopathic sideroblastic anemia (Gattermann et al. 1997). Two mutations have been described in association with *COX2*: (1) a heteroplasmic initiation codon mutation presenting with encephalopathy (Clark et al. 1999); and (2) a heteroplasmic missense mutation in a case of myopathy (Rahman et al. 1999). Four mutations have been described in *COX3*: (1) a 15 base pair microdeletion in a patient with myopathy and recurrent myoglobinuria (Keightley et al. 1996); (2) a heteroplasmic missense mutation in a patient with mitochondrial encephalopathy, lactic acidosis and stroke-like symptoms (MELAS) (Manfredi et al. 1995); (3) a nonsense mutation with a predicted truncation of the last 13 amino acids in a patient with encephalopathy and myopathy with lactic acidosis (Hanna et al. 1998); (4) a frameshift mutation resulting in Leigh-like syndrome (Tiranti et al. 2000).

COX deficiencies due to mutations in mtDNA generally have a disease onset that occurs in late childhood or adulthood (Shoubridge 2001). The unique nature of the mitochondrial genetics involved has made genotype-phenotype correlations all but impossible. Patients with identical alleles can have entirely different clinical presentations. This has made diagnosis difficult and treatment necessarily patient specific.

Primary COX deficiency caused by nuclear mutations

To date, no mutations have been described in nuclear genes that encode COX structural units (Shoubridge 2001). All diseases resulting from nuclear COX deficiency have been found to be due to secondary COX deficiencies.

Secondary COX deficiency caused by nuclear mutations

At present, only a small number of nuclear-encoded COX genes have been shown to be associated with human disease (Shoubridge 2001). The first assembly factor to be identified with a human COX deficiency was *SURF1* (Zhu et al. 1998). Deficiency of SURF1 results in an autosomal recessive, systemic neurodegenerative disorder known as Leigh Syndrome (LS) (Zhu et al. 1998). Patients with LS usually present in infancy with a subacute necrotizing encephalopathy (Zhu et al. 1998; Pecina et al. 2003; Pecina et al. 2004). This neurodegeneration is characterized by bilateral symmetrical necrotic lesions in the brainstem, basal ganglia, thalamus, and spinal cord (Zhu et al. 1998). *SURF1* was shown to be associated with LS by microcell-mediated chromosome transfer of chromosome 9 into patient fibroblasts where it rescued the COX deficiency. Deletion mapping further localized the LS locus to a 4.5 cM region of 9q34 and allowed the gene to be sequenced (Zhu et al. 1998). The subsequent expression of *SURF1* *c*DNA in patient fibroblasts was also observed to rescue the COX deficiency (Zhu et al. 1998). Over 40 pathogenic mutations have been described for *SURF1* (Pecina et al. 2004). Most of these mutations result in truncated proteins from nonsense mutations, but missense and splice site mutations have also been described (Pecina et al. 2004). A patient with a mutation in *SURF1*, who presented clinically with villous atrophy and hypertrichosis has

also been described (Von Kleist-Retzow et al. 2001). This patient had none of the classic neurodegeneration seen with LS. *SURF1* cDNA from this patient contained a 6 base pair deletion at the start of exon 7. Samples of skeletal muscle and fibroblasts of this patient did not contain detectable levels of SURF1. The samples also had markedly decreased steady state levels of COX subunits and COX activity, consistent with typical LS. While it has been proposed that SURF1 is involved in COX assembly, the exact role of this protein remains to be determined. As well, further research is required to understand how the different mutations of *SURF1* result in distinct clinical phenotypes.

A distinct French-Canadian form of COX-deficient LS, from Saguenay-Lac St. Jean (LSFC), has also been described (Merante et al. 1993). In contrast to the marked systemic COX deficiency observed with LS, patients with LSFC have severely affected brain and liver tissues, while kidney and heart are normal. These patients also have recurrent episodes of acute and often fatal metabolic acidosis (Morin et al. 1993). LSFC is common to the Saguenay-Lac St. Jean region of Quebec where the carrier rate is 1/23, with an incidence of 1/2000 live births (Mootha et al. 2003). The gene for this disease maps to chromosome 2p16 (Lee et al. 2001) and was identified to be the Leucine-rich pentatricopeptide repeat cassette protein (LRPPRC) (Xu et al. 2004). The LRPPRC protein has been observed to localize to the mitochondria, cytoplasm and nucleus. It was also found to have a preference for binding single-stranded RNA. LRPPRC is a weak homolog of yeast Pet309p (Xu et al. 2004), a protein known to be involved with COX1 translation (Manthey and McEwen 1995). Fibroblasts from a patient with an A354V mutation in LRPPRC have reduced LRPPRC protein and decreased translation of COXI (Xu et al. 2004), suggesting that LRPPRC may have a role in *COX1* mRNA translation in

human cells, comparable to Pet309p function in yeast.

Mutations in the human mitochondrial copper metabolism pathway have also been described, in *SCO1* (Valnot et al. 2000) and *SCO2* (Papadopoulou et al. 1999). SCO2 deficiency commonly presents clinically as a fatal infantile hypertrophic cardiomyopathy and encephalopathy with severe lactic acidosis (Papadopoulou et al. 1999). It may also present as spinal muscular atrophy type I (Werdnig-Hoffmann Syndrome), which has an extremely poor prognosis – 95% of patients die due to respiratory failure by 17 months of age (http://www.neuro.wustl.edu/neuromuscular/synmot.html#wh). Patients have severe reductions of COX activity in heart and skeletal muscle, and mild reductions in liver and fibroblasts. Immunohistochemistry also shows a marked reduction of COX1 and COX2 in muscle biopsies. *SCO2* is inherited in an autosomal recessive manner and almost all patients identified have been compound heterozygotes and every patient described to date has had G1541A (E140K) as one of their alleles. This mutation is near the predicted CXXXC motif and may alter the copper-binding domain, but the underlying molecular pathogenesis remains unknown (Jaksch et al. 2001a; Jaksch et al. 2001b). A second phenotype has been described in a patient homozygous for the G1541A (E140K) allele (Jaksch et al. 2001). These patients present with delayed infantile onset of cardiomyopathy and neuropathy. Fibroblasts from these patients *in vitro* demonstrate increased copper uptake, suggesting that copper metabolism is affected. The increased copper uptake may also indicate a potential compensatory mechanism. This data, combined with the observation that $CuCl_2$ in the media rescues the COX deficiency of

patient fibroblasts, may provide a potential therapeutic target for patients with SCO2 deficiency (Jaksch et al. 2001).

Mutations in *SCO1* present as COX deficiencies with neonatal-onset hepatic failure, metabolic acidosis and encephalopathy (Valnot et al. 2000). All affected individuals have been compound heterozygotes for a 2-base pair deletion resulting in a frameshift mutation and premature stop codon in exon 2. The second allele, P174L, results in the transition of a highly conserve proline near the CXXXC copper-binding motif (Valnot et al. 2000). Genetic screening of patient samples suggests that mutations of *hSCO1* are not a common cause of COX deficiency (Horvath et al. 2000).

Deficiency of both *COX10* (Valnot et al. 2000; Antonicka et al. 2003) and *COX15* (Antonicka et al. 2003; Oquendo et al. 2004), which encode products of the heme A biosynthetic machinery, have also been implicated in human disease. Mutations in *COX10* present with a broad and heterogeneous range of clinical phenotypes. The first patient described presented clinically with tubulopathy and leukodystrophy (Valnot et al. 2000), whereas more recent patients have presented with anemia and classical LS, or anemia, sensorineural hearing loss and fatal infantile hypertrophic cardiomyopathy (Antonicka et al. 2003). All patients described to date have had reduced steady state levels of all COX subunits, with almost complete loss of COX2 (Valnot et al. 2000; Antonicka et al. 2003). The patients have also been observed to contain reduced levels of heme A, as determined by HPLC, and lowered COX activity in affected tissues (Valnot et al. 2000; Antonicka et al. 2003). It is unclear why reduced heme A results in such a marked reduction of COX2 compared to the other COX subunits, as heme A is known to be bound to COXI (Antonicka et al. 2003). No COX subcomplexes, such as those seen

for *sco1* and *surf1* mutants, were observed, suggesting that heme A is necessary for stability of COXI (S1), or its interaction with COXIV and COXVa (S2) (Antonicka et al. 2003). Functional complementation of a yeast *cox10* null by a N204K allele did not occur, in contrast to wild-type h*COX10*, which rescues the respiratory deficiency (Valnot et al. 2000). Over-expression of *COX10* in immortalized fibroblasts from the patients restored COX activity (Antonicka et al. 2003). A partial rescue was observed when a mouse chromosome with *COX10* was transferred into these same fibroblasts. As with *COX10*, deficiency of *COX15* presents with heterogeneous clinical phenotypes (Antonicka et al. 2003; Oquendo et al. 2004). *COX15* deficiency has been observed in one patient to cause early onset fatal hypertrophic cardiomyopathy (Antonicka et al. 2003), while another patient presented with symptoms and radiological features consistent with LS (Oquendo et al. 2004). Patients deficient in COX15 present with severely decreased heme A levels and COX activity in heart tissue and fibroblasts (Antonicka et al. 2003). Expression of *COX15* in patients' fibroblasts increased heme A content and rescued COX activity (Antonicka et al. 2003). As observed with *cox10* patients, no COX subcomplexes were observed in *cox15* patients' fibroblasts. This observation is consistent with COX10 studies that suggest heme A is required for stabilization of COXI (S1) and/or formation of S2.

Summary

The biogenesis of COX is a complex process requiring the coordinated interaction of gene products from two separate genomes, mitochondrial and nuclear. Assembly of COX requires not only the polypeptides of the complex itself, but also a host of ancillary

proteins, some of which are required to synthesize the prosthetic groups. We are starting to develop a rudimentary understanding of all the processes involved in generating a functional COX enzyme, and it is clear that a mutation in any requisite COX assembly factor results in a specific COX deficiency. Mutations have been described in six assembly factors that cause COX deficiency in humans, including *COX10*, *COX15*, *LRPPRC*, *SCO1*, *SCO2*, and *SURF1*. All of these assembly factors have yeast homologs and were, with the exception of *SURF1*, first identified in yeast. Much of our understanding of the molecular pathology of these disorders has been assisted by studies in *S. cerevisiae*, and in fact, identification and characterization in yeast have helped the identification of human homologs and the subsequent patient mutations. The latter point is particularly important since the molecular basis for the underlying COX deficiency in a large number of patients remains unknown. Continued studies of COX assembly will provide a model for assembly of other oligomeric complexes, offering insight into basic molecular processes and the pathogenic mechanisms by which human disease arises. This thesis describes the characterization of one such assembly factor, Cox16p, and the identification of its mammalian homologues.

Chapter 2: Materials and Methods

Strains and Media: Bacterial and Yeast

E. coli were grown in LB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.1% glucose) for liquid culture and LB plates (0.5% yeast extract, 1% tryptone, 0.5% NaCl, 0.1% glucose, 2% agar) containing ampicillin (Amp) (50 μg/mL; Sigma) for solid medium.

The yeast strains used in the experimental procedures include the wild-type yeast strains, W303 (α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and aW303 (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*), and the *cox16* null mutant strain, W303ΔCOX16 (α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox16::URA3*) and aW303ΔCOX16 (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox16::URA3*). The *COX16* gene was disrupted by insertion of a 1.1-kb *URA3* fragment at the internal *Hin*dIII site in *COX16*. The ρ^o tester strains used were KL14ρ^o (α *met6*) and CB11ρ^o (*a ade1*).

YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) is a rich glucose medium that was used as a solid growth medium for yeast. Strains were cultured in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose) for yeast transformations. After the yeast transformations, cells were plated on WO medium (0.67% nitrogen base without amino acids, 2% glucose, 2% agar) that was supplemented with the appropriate amino acids (combinations of histidine, adenine, uracil, trytophan). To test for respiratory competence, cells were grown on ethanol/glycerol (EG) (1% yeast extract, 1% peptone, 2% ethanol, 2% glycerol, 2% agar). To isolate mitochondria, cells were grown in Galactose (GAL) medium (1% yeast extract, 1% peptone, 2% GAL) to induce mitochondrial proliferation.

DNA Clones and Constructs

Construction of pMGL5 and the COX16-Myc Fusion

To overexpress a Cox16p-Myc fusion protein, I first constructed pMGL5, a yeast/*E. coli* shuttle plasmid containing a Myc epitope tag (EQKLISEEDL), in the backbone of the multicopy vector, YEp351 (Hill et al. 1986). Briefly, a 3.3-kb *Nar*1-*Aat*II fragment of YEp351, containing the *LEU2* marker and the 2-μm origin of replication, was used to replace a 3.8-kb *Nar*1-*Aat*II fragment in YCpMyc111 (a gift from Dr. Troy Harkness, modified from Gietz and Sugino (Gietz and Sugino 1988)). This converted the CEN plasmid, a low copy plasmid, into an episomal plasmid, which persists at a high copy number. To make the Myc fusion, *COX16* was amplified by PCR, using primers that introduced a *Pst*I site 216 nucleotides upstream of the start codon (5'-gttattagactgcagatacacttcc-3') and a *Sma*I site two nucleotides upstream of the termination codon (5'-cgttttgaatgttcccgggcattc-3'). The 416-bp PCR product was ligated to pMGL5, resulting in an in-frame fusion of *COX16* to the Myc epitope. The same *COX16* fragment was also fused to the Myc sequence in the CEN plasmid YCpMyc111. Both constructs were verified to be in-frame by automated sequencing (LiCor, Lincoln, NE).

Construction of pCOX16H/ST1 with ADH promoter

The hCOX16 gene (HSPC203) was amplified by PCR from a HeLa cDNA library using the primers HSPC203F (5'-ggtgagagcgtgagctcctgagatttggg-3'), which introduced a *Sac*I restriction site 62 bp upstream of the start codon, and HSCP203R (5'-ggaaaaaagatctgcagagtcaagttgtc-3'), which introduced a *Pst*I restriction site 3 bp

downstream of the stop codon. The 420-bp PCR product was ligated into pMGL3, which contains an ADH1-promotor and a LEU2 prototrophic selectable marker. The resulting plasmid sequence was verified by automated sequencing (LiCor, Lincoln, NE) using the primers PMGL-F and ADH-T.

Molecular Biology Techniques

Transformation of competent E. coli cells

E. coli cells were made competent by the method of Inoue *et al.* (Inoue et al. 1990) and stored in aliquots at -80°C. Cells were thawed at room temperature and 200 µL placed in a sterile microcentrifuge tube on ice. 5-10 µL of plasmid DNA were added to the competent cells, followed by incubation for 30 minutes on ice. The cells were then heat-shocked at 42°C for 30 seconds and subsequently placed on ice for two minutes. Cell were mixed with 0.8 mL of liquid LB medium in a sterile glass tube and incubated at 37°C for 30-60 minutes, with shaking (225 rpm). 50 µL of this cell culture was then plated on selective LB agar plates and incubated at 37°C overnight.

***E.coli* Plasmid MiniPrep**

Individual bacterial colonies were streaked in small patches (approximately 1 cm^2) on selective LB agar plates and incubated overnight at 37°C. Cells were collected using a sterile toothpick and resuspended in sterile microcentrifuge tubes that contained 100 µL of cold lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 5mg/mL lysozyme, 0.02 mg/mL RNAse A). Cells were incubated on ice for one minute followed by the addition of 200 µL of 0.2 M NaOH, 1% SDS and mixing by inversion. 150µL of 7.5 M ammonium acetate was then added to the tubes and mixed by shaking.

Suspensions were centrifuged at 14,000 rpm for 5 minutes in a microcentrifuge. The supernatant was then decanted into a fresh 1.5 mL microcentrifuge tube that contained 300 µL of isopropanol and 0.2% Triton X-100. The tubes were mixed by shaking and centrifuged for 5 minutes at 14,000 rpm. The plasmid DNA was washed with 80% ethanol/0.2 mM EDTA, followed by a second wash with 80% ethanol. Plasmid DNA was dried in the SpeedVac Plus (Savant) for 5 minutes and resuspended in 20-50 µL of water.

Purification of DNA Fragments with Glass Milk

DNA was electrophoresed in 1% agarose gels. The fragment of interest was excised from the gel, placed in a sterile 1.5 mL microcentrifuge tube, and weighed. Three volumes of NaI (22.7 gm NaI dissolved in 25 mL ddH_2O, to which 0.38 gm Na_2SO_3 was added) were added to the excised DNA and incubated at 65°C until the agarose had melted (typically 5-10 minutes). 10-15 µL of glass milk (made with crushed silica) were added to the suspension and incubated at room temperature for 10 minutes and vortexed every two minutes. The suspension was then centrifuged at 7,000 rpm to pellet the glass milk. The supernatant was aspirated and the pellet washed three times with 700 µL of NEET solution (100 mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.5, 50% ethanol). The glass milk pellet was resuspended with 10-15 µL of sterile water, vortexed to mix, and incubated at 65°C for 10 minutes. The suspension was centrifuged at 14,000 rpm for one minute and the DNA-containing supernatant transferred to a fresh tube. The elution of DNA was then repeated on the glass milk pellet. The elutions were pooled, and the recovery of DNA was quantitated by electrophoresis on a 1% agarose gel.

Site-Directed Mutagenesis of Yeast *COX16*

Specific mutations were introduced in yeast *COX16* using the Quickchange Site-Directed Mutagenesis Kit (Stratagene). Site-directed mutagenesis reactions were carried out according to the manufacturer's manual, using 10 ng of pCox16/St5 as template DNA. pCox16/St5 is Sc*COX16* in the low copy plasmid YCp352. The primers used in the site-directed mutagenesis reactions are listed in Table 2-1. The PCR reaction was 95°C for 30 sec, followed by 16 cycles of 95°C for 30 sec, 55°C for 1 min, 68°C for 7 min, and then 4°C for 5 min. 0.5 µL DpnI was then added for 1 hour at 37°C. Plasmids carrying mutations were isolated by the Triton Plasmid DNA preparation method (see next section) and sequenced using the AB3100 Avant capillary sequencer (Applied Biosystems). The resulting plasmids were transformed into the *cox16* null mutant strain, selected for Leu prototrophy on WO medium, and replica-plated to EG medium to assess respiratory growth.

Triton Plasmid DNA Preparation

An *E. coli* strain carrying a plasmid of interest was streaked over the entire surface of an LB+AMP plate and incubated at 37°C overnight. The lawn of cells was scraped using a sterile spatula and placed into a 15 mL borosilicate tube containing 1 mL of cold buffer (5% sucrose, 50 mM Tris pH 8.0). One mL of lysozyme buffer (4.5 mg/mL lysozyme, 22.7 mM Tris pH 8.0, 113.6 mM EDTA, 0.9 mg/mL RNase A) was added to the cell suspension, the tube mixed by inversion, and then incubated on ice for 30 minutes. One mL of cold Triton X-100 lysis buffer (0.3% Triton X-100, 0.185 M EDTA, 0.15 M Tris

Table 2.1 Primer pairs used in the site-directed mutagenesis of yeast *COX16*

Mutation	Forward Primer	Reverse Primer
V46F	Cox16F V46F GTGCAACAATA**TTTTT**GGGTTCGTTTTGG	Cox16R V46F CCAAAACGAACCCAAAAATATTGTTGCAC
L47F	Cox16F L47F GTGCAACAATAGTA**TTT**GGTTCGTTTTGG	Cox16R L47F CCAAAACGAACCAAA**T**ACTATTGTTGCAC
G48F	Cox16F G48F GTGCAACAATAGTATTG**TTT**TCGTTTTGG	Cox16R G48F CCAAAACGAA**AA**CAATACTATTGTTGCAC
L52F	Cox16F L52F GGGTTCGTTTTGGTT**T**TCAAGCTTTACAGCG	Cox16R L52F CGCTGTAAAGCTTGA**A**AACCAAAACGAACCC
K59E	Cox16F K59E GCTTTACAGCGATC**G**AGTACGAGCAAGGCG	Cox16R K59E CGCCTTGCTCGTAC**T**CGATCGCTGTAAAGC
Y60A	Cox16F Y60A GCTTTACAGCGATCAAG**GC**CGAGCAAGGCG	Cox16R Y60A CGCCTTGCTCG**GC**CTTGATCGCTGTAAAGC
E61K	Cox16F E61K CAGCGATCAAGTAC**A**AGCAAGGCGATCG	Cox16R E61K CGATCGCCTTGCT**T**GTACTTGATCGCTG
E73K	Cox16F E73K CAGGAAATTAATGAAA**A**GGATATCTTG	Cox16R E73K CAAGATATCCTTTTCATTAATTTCCTG
R83E	Cox16F R83E GGAAGAATCAA**GA**GGAATTTGATATTAAAG	Cox16R R83E CTTTAATATCAAATTCC**T**CTTGATTCTT
E90K	Cox16F E90K GATATTAAAGAAA**A**ATATTATCGTTTACAAGG	Cox16R E90K CCTTGTAAACGATAATATTTTTCTTTAATATC
Y91D	Cox16F Y91D GATATTAAAGAAGAA**G**ATTATCGTTTACAACG	Cox16R Y91D CCTTGTAAACGATAAT**C**TTCTTCTTTAATATC
R93D	Cox16F R93D GATATTAAAGAAGAATATTAT**GA**TTTACAAGGTC	Cox16R R93D GACCTTGTAAA**TC**ATAATATTCTTCTTTAATATC
L94F	Cox16F L94F GAAGAATATTATCGTTT**T**CAAGGTCTTTCTG	Cox16R L94F CAGAAAGACCTTGA**A**AACGATAATATTCTTC
E100K	Cox16F E100K GGTCTTTCTGAAAAGGATTGGGAGCC	Cox16R E100K GGCTCCCAATCCT**T**TTCAGAAAGACC
W102A	Cox16F W102A CTGAAGAGGAT**GC**GGAGCCTGTGCGCG	Cox16R W102A CGCGCACAGGCTC**C**GCATCCTCTTCAG
R106D	Cox16F R106D GGGAGCCTGTG**GA**CGTCGCTAGGTTAAAGG	Cox16R R106D CCTTTAACCTAGCGACG**TC**CACAGGCTCCC
V107F	Cox16F V107F GGGAGCCTGTGCGC**T**TCGCTAGGTTAAAGG	Cox16R V107F CCTTTAACCTAGCGAAGCGCACAGGCTCCC
R109E	Cox16F R109E GCCTGTGCGCGTCGCT**GA**GTTAAAGGATGAATC	Cox16R R109E GATTCATCCTTTAAC**TC**AGCGACGCGCACAGGC

Mutated bases shown in bold.

pH 8.0) was added to the tubes, which were mixed by inversion. The cell mixture was transferred to a polyallomer thick wall ultracentrifuge tube (13 X 56 mm; Beckman), balanced, and centrifuged at 40,000 rpm for 20 minutes at 4°C in an Optima TLX table-top ultracentrifuge (Beckman). The supernatant was transferred to a new 15 mL conical tube, which then had an equal volume of water-saturated phenol added to it. The tubes were mixed by vortexing and then centrifuged at 4000 rpm for 5 minutes at 4°C in a J6-MI centrifuge (Beckman). The upper aqueous phase was transferred into a new 15 mL conical tube. The tube was filled with ether and mixed by shaking. Once the two phases had separated, the top layer was aspirated. The ether wash was repeated until the lower aqueous phase was clear. Once the lower phase was clear, 0.05 volumes of 5 M NaCl and three volumes of 100% ethanol were added and the suspension mixed by inversion. The tube was centrifuged at 4,000 rpm for 10 minutes at 4°C, forming a small, oily pellet. The supernatant was aspirated and the pellet dissolved in 2 mL 2 M ammonium acetate, followed by the addition of three volumes of 100% ethanol. The tube was centrifuged at 4,000 rpm for ten minutes at 4°C, resulting in a white DNA pellet. This pellet was dissolved in 300 µL of 2 M ammonium acetate and transferred to a 1.5 mL microcentrifuge tube. After adding 2.5 volumes of ethanol, the tube was centrifuged for 5 minutes at 14,000 rpm in a microcentrifuge. The DNA pellet was washed once with 80% ethanol, 0.2 mM EDTA and then with 80% ethanol. The pellet was dried in a SpeedVac for approximately 5 minutes and resuspended in 30-50 µL of water. The concentration of the plasmid DNA was determined using DNA95 software on a Shimadzu UV-Visible UV-1601 PC spectrophotometer.

Yeast Transformation by Lithium Acetate Method

The following protocol is a modification of the method described by Schiestl and Gietz (Schiestl and Gietz 1989). Yeast were inoculated into 10 mL YPD and grown overnight at 30°C, with shaking (225 rpm). 2 mL of the overnight culture was inoculated into a sterile 250 mL flask containing 100 mL of fresh YPD. Cells were grown at 30°C with shaking for 3-4 hours until the culture OD_{600} was between 0.6 and 1.0. The culture was then centrifuged at 2,500 rpm, for 5 minutes at room temperature, in sterile 50 mL conical tubes. The supernatant was poured off and the pellets were resuspended in 10 mL TEL (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M LiAc) and centrifuged at 2,500 rpm for another 5 minutes. The supernatant was poured off and the pellets resuspended in 0.9 mL TEL. 100 µL aliquots of the cells were placed in sterile microcentrifuge tubes. To each aliquot, 5-10 µl of transforming DNA and 10 µL of salmon sperm carrier DNA (10 mg/mL; denatured at 90°C for 10 minutes) were added. After incubating for 30 minutes at room temperature, 0.7 mL of PEG/TEL buffer (40% polyethylene glycol, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M LiAc) was added and mixed well. This mixture was incubated for 45-60 minutes at room temperature, before cells were heat shocked at 45°C for 10-15 minutes. Cells were then centrifuged at 7,000 rpm for 10 seconds. After removing the supernatant, the pellets were resuspended in 0.2 mL TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and centrifuged again for 10 seconds. The supernatant was discarded and the pellets resuspended in 50 µl TE and spread on minimal medium (WO) to select for specific prototrophies. Yeast colonies were observable on plates after incubating for three days at 30°C.

Biochemical Techniques

Preparation of Intact Yeast Mitochondria by Zymolyase

Ten mL of liquid GAL were inoculated with a yeast strain and incubated overnight at 30°C with shaking (225 rpm). Two mL of the overnight culture were used to inoculate 50 mL of liquid GAL, which was incubated overnight at 30°C with shaking (225 rpm). 25 mL of this overnight culture were used to inoculate 800 mL GAL, which was incubated overnight at 30°C with shaking (225 rpm). Cells were harvested by pouring culture into a 1 L centrifuge bottle and centrifuging at 2,000 rpm for 5-7 minutes in a Beckmen J6-MI centrifuge. After pouring off the medium, the cells were resuspended in 1.2 M sorbitol and transferred to a pre-weighed 250 mL centrifuge bottle. The cells were then centrifuged at 6,000 rpm for 5 minutes at 4°C in a SLA-1500 rotor in a Sorvall RC-5B centrifuge. The supernatant was poured off, the wet weight determined and the cells then resuspended in 30 mL digestion buffer (1.2 M sorbitol, 0.075 M NaP_i pH 7.5, 1 mM EDTA, 0.1% ß-mercaptoethanol, 0.5 mg/mL Zymolyase, (Seikagaku)) per 10 gms of cells (wet weight). Cells were incubated at 37°C until most of the cells had been converted to spheroplasts (typically 60-90 minutes for respiratory deficient strains and 2-3 hours for wild-type strains). After digestion, 1.2 M sorbitol was added to the centrifuge bottle to a final volume of 200 mL. The cells were centrifuged at 6000 rpm for 10 minutes at 4°C. Cells were washed twice more with sufficient buffer (150 mL-200 mL) to remove excess Zymolyase. The spheroplasts were then resuspended in STE buffer (0.5 M sorbitol, 20 mM Tris pH 7.5, 0.5 mM EDTA) at a concentration of 30-50 mL per 10 grams of starting cells. The spheroplasts were homogenized in a Waring blender for 20 seconds. 100 µL of phenyl methylsulfonyl fluoride (PMSF) (10 mg/mL)

were added to the homogenized spheroplasts, which were then poured into 50 mL tubes. The centrifuge tubes were centrifuged at 2,500 rpm for 10 minutes at 4°C using a SS-34 rotor (Sorvall). The supernatant was collected and centrifuged again at 2500 rpm for 10 minutes at 4°C to ensure that all cellular debris was cleared from pellets. The cleared supernatant was then transferred to a new tube and centrifuged at 12,000 rpm for 15 minutes at 4°C to sediment the mitochondria. The post-mitochondrial supernatant (PMS) was collected and kept on ice. The mitochondrial pellet was washed three times with STE by centrifugation at 12,000 rpm for 10 minutes at 4°C each time. After being washed, the mitochondria were resuspended in 200-500 µl 20 mM Tris, pH 7.5. 10 µL PMSF (10 mg/mL) were added to both the PMS and resuspended mitochondria. One mL of the PMS fraction was centrifuged in a polyallomer thick wall ultracentrifuge tube (13 X 56 mm; Beckmen) at 40,000 rpm for 20 minutes at 4°C to sediment any broken mitochondria. The PMS was transferred to a new tube.

Preparation of Intact Yeast Mitochondria with Intact Outer Membranes

Mitochondrial preparations used for sub-mitochondrial localization were isolated essentially as described by Glick (Glick 1995). The protocol is as described in 'Preparation of Intact yeast mitochondria by Zymolyase', up to the point of spheroplast formation by Zymolyase. After digestion, buffer A (deionized 1.2 M sorbitol, 20 mM KPO_4, pH 7.5) was added to the centrifuge bottle to a final volume of 200 mL. The cells were centrifuged at 6,000 rpm for 10 minutes at 4°C. Cells were washed twice more with sufficient buffer (150 mL-200 mL) to remove all the Zymolyase. The spheroplasts were resuspended in buffer B (0.6 M sorbitol, 20 mM KMES (potassium salt of MES) pH 6.0, 0.5 mM PMSF) at a concentration of 30-50 mL per 10 gms of starting cells and then

homogenized with a loose-fitting Teflon pestle for 30 strokes and then poured into 50 mL centrifuge tubes. The tubes were centrifuged at 3,500 rpm for 5 minutes at 4°C in a SS-34 rotor (Sorvall). The cleared supernatant was then transferred to a new tube and centrifuged at 10,000 rpm for 10 minutes at 4°C to sediment the mitochondria. The mitochondrial pellet was resuspended in buffer C (0.6 M sorbitol, 20 mM KMES pH 7.4) by gently homogenizing. The tubes were centrifuged again at 3,500 rpm for 5 minutes at 4°C. The cleared supernatant was then transferred to a new tube and centrifuged at 10,000 rpm for 10 minutes at 4°C. The post-mitochondrial supernatant (PMS) was collected and kept on ice. The mitochondrial pellet was resuspended in 1 mL KMES and diluted with 30 mL of buffer D (0.6 M sorbitol, 20 mM HEPES pH 7.4) and centrifuged at 10,000 rpm for 10 minutes at 4°C. After being washed, the mitochondria were resuspended in buffer D.

Determination of Protein by Folin Procedure

Protein concentrations were determined by the method of Lowry *et al.* (Lowry et al. 1951). In separate glass test tubes (13 x 100 mm), either 5 µL mitochondrial suspension + 595 µL water or 10 µL post-mitochondrial supernatant + 590 µL water were added. 600 µl of water was used as a reagent blank. To each sample, 3 mL of copper reagent (0.5 mL 1% $CuSO_4$, 0.5 mL 2% NaK tartate, and 49 mL 2% Na_2CO_3 in 0.01 N NaOH) was added, vortexed and incubated at room temperature for 10 minutes. 0.3 mL of Folin reagent (1:1 dilution of stock, Sigma) was then added to each tube and mixed immediately. Samples were heated at 90°C for 2 minutes, then placed on ice to cool and re-equilibrate to room temperature. Absorbance was read at 750 nm versus the reagent blank using a Shimadzu UV-Visible UV-1601 PC spectrophotometer.

Sucrose Gradient Density Centrifugation

Density gradient centrifugation was carried out using purified mitochondria (3 mg) from strain aW303ΔCOX16/Myc1, which were extracted with 1% deoxycholate and 0.5 M NaCl and loaded onto 2.4 mL 7-20% sucrose gradients (7% or 20% sucrose, 0.1% Tx-100, 10 mM Tris-HCl, pH 7.5, and 0.5 M NaCl) (Glerum et al. 1995). 0.3 mg lactate dehydrogenase (LDH) (5 mg/mL (3.2 M) in $(NH_4)_2SO_4$ was centrifuged for 10 minutes, then the pellet was resuspended in 50 µL 30 mM NaPi, pH 7.5), along with 2.5 mg hemoglobin (Hb) (2.5 mg in 50 µL H_2O), were added to the extract as internal standards. Gradients were chilled at 4°C for 1 hour before addition of the extract and, following loading of the samples, centrifuged for 12 hours at 4°C in a Beckman Optima TLX table-top ultracentrifuge at 54,000 rpm with the brake turned off. 4 drops per fraction were collected into 14-16 Eppendorf tubes. 5-10 uL of 200 mM PMSF were added to Eppendorf tubes immediately before collecting samples. Hb levels were determined by adding 25 µL of each fraction to 800 µL water and measuring absorbance at 410 nm on the Shimadzu UV-Visible UV-1601 PC spectrophotometer. Fractions were prepared for LDH analyses by mixing 4-5 µL of sample with 30 µL 10 mM pyruvate, 100 µL 2 mM NADH and 860 µL 30 mM NaPi (pH 7.5). Activity was measured for 30 sec at 340 nm on the Shimadzu UV-Visible UV-1601 PC spectrophotometer. The molecular weight of the native complex was estimated using the calculation (R = distance to meniscus (unknown)/distance to meniscus of standard; $R=S_1/S_2$; $S_1/S_2=(MW_1/MW_2)^{2/3}$) (Martin and Ames 1961).

Yeast Mitochondrial Cytochrome Spectra

Mitochondrial cytochromes were extracted by dissolving 100 mg KCl in deionized water and mixing with 100 µL Tris-HCl (1 M, pH 8.0), then adding 13 mg of mitochondria, and 200 µL 10% deoxycholate to a final volume of 2 mL. The sample was centrifuged in a polyallomer thick wall ultracentrifuge tube at 40,000 rpm for 20 minutes at 4°C. The supernatant was transferred to a 13 X 100 mm borosilicate tube (Fisher), followed by the addition of 100 µL 20% cholate. The contents of the tube were mixed by inversion and split between two cuvettes, which were placed in the rear and front cell holders of a Shimadzu UV-Visible UV-1601 PC spectrophotometer. The sample in the rear cuvette was oxidized with 100 µL KFeCN (Sigma). The sample in the front cuvette was reduced with $Na_2S_2O_4$ (dithionite) (Sigma). The oxidized minus reduced spectra of the extracted cytochromes was recorded using the "Spectrum" mode of the UV Probe Shimadzu software program. All spectra from transformed yeast strains were recorded at the same time as spectra from a wild-type yeast strain and a *cox16* null strain.

Western Blotting

Protein extracts were prepared in sample buffer (4X stock: 0.19 M Tris-HCl pH 6.8, 3.85% SDS, 0.04% β-mercapoethanol, 38.5 % glycerol, 0.02-0.05% bromophenol blue w/v) and separated by 12% (Laemmli 1970) or 15 % with glycerol (Glerum and Tzagoloff 1998) SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Both 12% and 15% gels were run at 150 V for 1.5 hours. After electrophoresis, proteins were transferred to nitrocellulose (Trans-Blot Transfer Medium, BIO-RAD) using the Mini-Protean II system (BIO-RAD). The transfer was carried out at

100 V for 60 minutes in cold transfer buffer (200 mM glycine, 25 mM Tris-HCl, and 20% methanol). Following the transfer, the nitrocellulose membrane was stained with Ponceau S (2% Ponceau S (Sigma), 30% trichloroacetic acid, and 30% sulfosalicylic acid) for 5 minutes and then rinsed with water. After visualization, the protein standards were marked. Membranes were then placed on a rocker platform for 60 minutes in blocking buffer (3% powdered milk in rinse buffer (RB)(140 mL 1M Tris pH 8.0, 28 mL 0.5 M EDTA, 420 mL 5M NaCl, 140 mL 10 Triton X-100). The blot was then placed in fresh blocking buffer containing primary antibody (Table 2-2) and incubated overnight at 4°C with rocking. The blot was then washed three times in RB + salt (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100) for 10 minutes with rocking. The blot was placed in fresh blocking buffer containing secondary antibody conjugated to horseradish peroxidase (goat anti-mouse or goat anti-rabbit antibody; Transduction Laboratories) and incubated for 1 hour with rocking, followed by three washes in rinse buffer for ten minutes with rocking. Proteins were detected using the Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagents (Amersham Biosciences). The blot was wrapped in Saran Wrap and exposed to Kodak X-OMAT or BioMax film, which was developed using a M35A X-OMAT processor (Kodak).

Tricine-SDS-Polyacrylamide Gel Electrophoresis for the Separation of Low Molecular Weight Proteins

The 16.5% polyacrylamide gel system used to separate low molecular weight proteins (in the range of 5-20 kDa) is based on the method described by

Table 2.2 Primary antibodies used in this study.

Primary Ab	Company	Dilution	Secondary
Anti-Myc	Sigma	1/2000	Anti-Mouse
Anti-Myc	Molecular Probes	1/2000	Anti-Mouse
Anti-Porin	Molecular Probes	1/5000	Anti-Mouse
Anti-CO20 (Cox1p)	N/A	1/2000	Anti-Rabbit
Anti-CO29 (Cox2p, Cox4p, Cox5p, Cox6p)	N/A	1/2000	Anti-Rabbit
Anti-Cox3p	Molecular Probes	1/2500	Anti-Mouse
Anti-Sco1	N/A	1/2500	Anti-Rabbit

(Schägger and von Jagow 1987). To prepare the separation gel, 5 mL of acrylamide stock B (46.5% acrylamide, 3% bis-acrylamide), 5 mL gel buffer (GB)(3 M Tris-HCl pH 8.45, 0.3% SDS), and 5.4 gm urea were mixed with water to a final volume of 15 mL. The separation gel was polymerized by adding 50 µL 10% ammonium persulfate (APS; GibcoBRL) and 7.5 µL TEMED (GibcoBRL) and then over-layed with water. After the separation gel had set, the water was removed and the spacer gel was poured so that it was one-sixth the volume of the separation gel. The spacer gel consisted of 2.03 mL of acrylamide stock A (48% acrylamide, 1.5% bis-acrylamide), 3.33 mL GB and water to a final volume of 10 mL. The spacer gel was polymerized by adding 33.3 µL 10% APS and 3.33 µL TEMED and then overlayed with water. The water was removed once the spacer gel had set. The stacking gel was prepared using 0.5 mL acrylamide stock A, 1.55 mL GB and water to a final volume of 6.25 mL. Protein extracts were prepared in 1X sample buffer (4% SDS, 12% glycerol, 50 mM Tris-HCl pH 6.8, 2% ß-mercaptoethanol, 0.01% Serva blue G). Separate 1X anode (10X stock: 2 M Tris-HCl pH 8.9) and 1X cathode (10X stock: 1 M Tris, 1 M Tricine, 1% SDS; pH 8.25) buffers were used during electrophoresis. Gels were electrophoresed for 3.5 hours at 100 V.

Metal Salt Supplementation Protocol

Metal enriched EG plates were generated to test for suppression of the *cox16* null. The metal salts tested were $CuSO_4$, $MnSO_4$, $ZnSO_4$, $FeSO_4$, $MgSO_4$, and $CaCl_2$. 10% w/v stock solutions were made in ddH_2O for each salt and then filter sterilized. EG was distributed at 50 mL/flask and then autoclaved. The required amount of metal solution was mixed with H_2O in a sterile Falcon tube to a total volume of 10 mL. The

concentrations tested were 0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.4%, and 0.8% (w/v) (in 60 mL total). Each solution was then added to the molten EG, mixed well, and poured. Plates were then allowed to dry on the bench over two nights.

HPLC Analysis of Mitochondrial Hemes

Total heme was extracted from 3 mg of purified mitochondrial protein with 1 mL of acetone containing 2.5% HCl. The mixture was vortexed, clarified by centrifugation and mixed with an equal volume of 50% acetonitrile. Insoluble material was removed by a second centrifugation. The extract was adjusted to approximately pH 3.5 with 1.65 M ammonium hydroxide, clarified by centrifugation, and applied to a 3.9 × 300 mm C18 Bondclone column (Phenomenex, CA, USA). Hemes were eluted at a flow rate of 1 mL/min using a 30–50% acetonitrile gradient over the first 5 mL, followed by a 50–75% linear acetonitrile gradient over the following 35 mL. The acetonitrile solutions contained 0.05% trifluoroacetic acid. The elution of heme compounds was monitored at 400 nm. Elution times are affected by the pH of the sample applied to the column.

Chapter 3: Results of COX16 Characterization

Portions of the material from this chapter have been published in:

Carlson CG, Barrientos A, Tzagoloff A, Glerum DM. (2003) *COX16* encodes a novel protein required for the assembly of cytochrome oxidase in *Saccharomyces cerevisiae*. J. Biol. Chem., 278: 3770-3775.

***cox16* Mutants Are Defective in Cytochrome Oxidase Assembly**

A catalogued collection of nuclear genes necessary for *S. cerevisiae* respiration (*pet* mutants) has been generated by the Tzagoloff lab (Dr. A. Tzagoloff, Columbia University, New York). *S. cerevisiae* from complementation group G22 of this *pet* mutant collection (Tzagoloff and Dieckmann 1990) are respiration-deficient due to a specific loss of COX. Spectral analyses of mitochondria isolated from G22 mutants showed a partial or complete loss of the 605-nm cytochrome aa_3 peak. Since the respiratory defect could be complemented by ρ^0 mutants, the COX deficiency was due to recessive mutations in a nuclear gene, which was subsequently designated *COX16*. To identify the gene responsible for the COX deficiency of G22 mutants, C25/U1 (a uracil auxotrophic derivative of the G22 mutant, C25) was transformed with a yeast genomic library and uracil prototrophic clones were checked for growth on non-fermentable carbon sources. Subcloning revealed an open reading frame (ORF) that was identical to ORF YJL003W on chromosome X (GenBank accession number Z49278) in the yeast genome. In keeping with the naming of the genes for COX assembly factors, the ORF was designated *COX16*. Subsequent disruption of the *COX16* gene confirmed that the mutants in complementation group G22 have mutations in the *COX16* gene. The biochemical phenotype of aW303ΔCOX16 was determined to be similar to that of the G22 mutants studied previously in the Tzagoloff lab. It displays a selective absence of cytochromes aa_3 and a loss of COX activity (Figure 3.1).

As demonstrated for other COX assembly factor mutants (Glerum et al. 1997), the steady state levels of Cox1p, Cox2p and Cox3p are markedly reduced in the *cox16* null

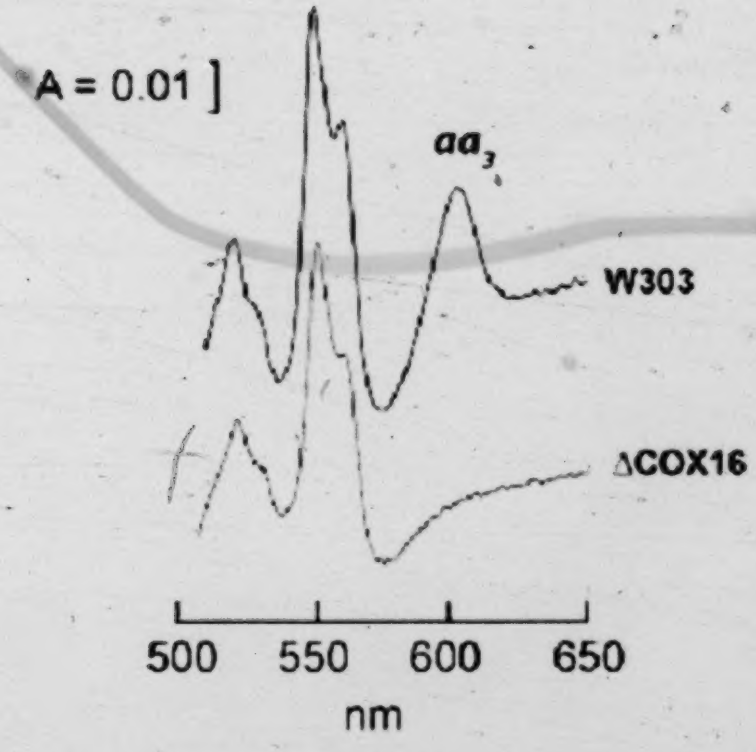

Figure 3.1. Spectral analysis of mitochondrial cytochromes. Mitochondria (6.5 mg/mL) were prepared from the wild type strain W303-1A (W303) and from the *cox16* mutant aW303ΔCOX16 (Δ*COX16*). The position of cytochrome aa_3 is marked.

mutant (Figure 3.2). The nuclear-encoded subunits are present at normal concentrations with the exception of Cox5p, which appears to be slightly decreased. *In vivo* labeling of C25, in the presence of cycloheximide to block cytoplasmic protein synthesis, had indicated that the three mitochondrially-encoded subunits of COX are correctly expressed, although labeling of Cox2p appeared to be slightly lower than in wild type. To determine if Cox16p participates in export of the Cox2p precursor, the size and stability of this mitochondrial gene product was compared in different COX null mutants with identical nuclear backgrounds by Dr. Antoni Barrientos (Columbia University, New York, USA). The results of the *in vivo* pulse-chase labeling experiment confirmed that only fully processed Cox2p is detected in the *cox16* null mutant aW303Δ*COX16* after the 15-min pulse (Figure 3.3). Since proteolytic cleavage of the pre-sequence requires export of the amino-terminal transmembrane domain to the IMS (Hell et al. 1997), these results exclude Cox16p from having a function in membrane insertion of the N-terminal domain of the precursor. The pulse-chase results also make it unlikely that Cox16p is involved in membrane insertion of the C-terminal domain of Cox2p. By comparison, the turnover of Cox2p in a *cox18* mutant was greatly increased compared with the wild type or other COX mutants (*e.g. cox15*). Cox18p is required for the export of the carboxy-terminal domain of Cox2p (Saracco and Fox 2002), and the rapid degradation of the protein probably occurs as a consequence of exposure of the carboxy-terminus to proteolytic enzymes found in the IMM and/or matrix. In contrast, the *cox15* and *cox16* mutants were observed to have a similar Cox2p turnover rate during the 30-min chase (Figure 3.3). Since Cox15p functions in heme A synthesis (Barros et al. 2002), the accelerated turnover rate of Cox2p in *cox15* mutants is unlikely to be due to a problem with

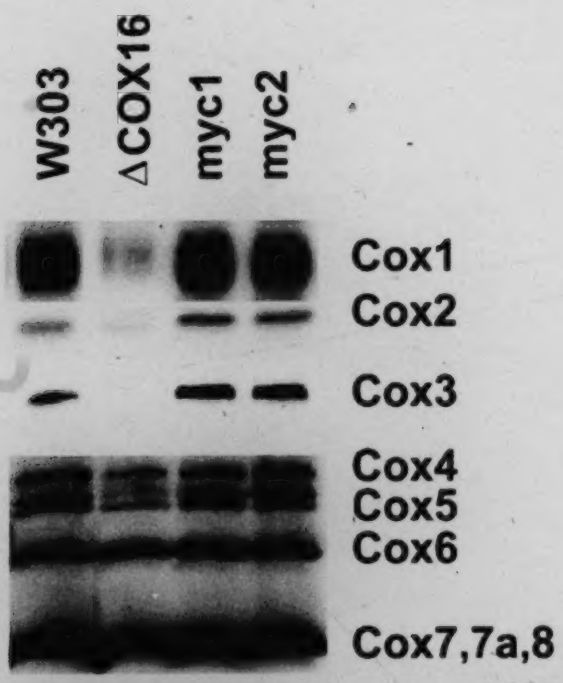

Figure 3.2. Western analysis of COX subunits. Mitochondria were isolated from the wild type W303-1A (W303), the mutant aW303ΔCOX16 (ΔCOX16), aW303Δ COX16/Myc1 (Myc1) and aW303ΔCOX16/Myc2 (Myc2), which are the *cox16* mutant transformed with the *COX16-Myc* fusion in a high-copy and a CEN plasmid, respectively. Subunits 1, 2, and 3 of cytochrome oxidase (Cox1, Cox2, Cox3) were analyzed by separating 10 μg of mitochondrial protein on 12% polyacrylamide gels. The nuclear-encoded subunits (Cox4p; Cox5p; Cox6p; Cox7p, Cox7ap, Cox8p) were detected by separating 20 μg of mitochondrial protein on a 16.5% polyacrylamide/6 M urea gel. Following transfer to nitrocellulose, the blots were probed with subunit-specific antibodies and visualized using enhanced chemiluminescence.

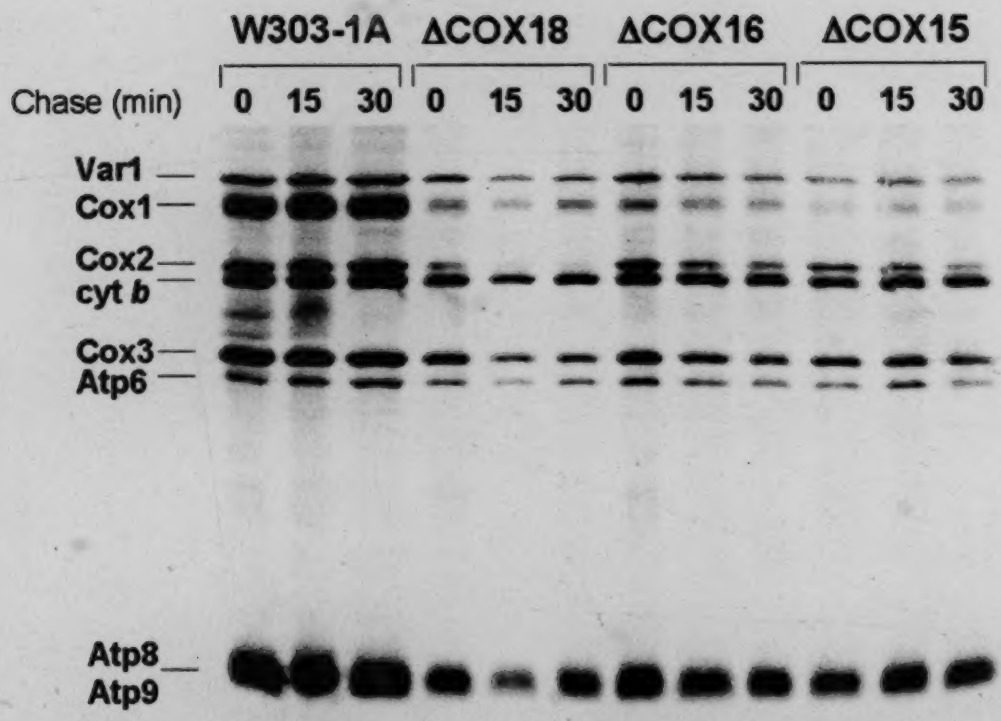

Figure 3.3. Turnover of *in vivo* labeled mitochondrial translation products in wild type and *cox16* mutants. The parental wild type (W303-1A), a *cox18* null mutant (Δ *COX18*), a *cox16* null mutant (ΔCOX16), and a *cox15* null mutant (COX15) were analyzed. The identities of the labeled proteins are marked in the margin: ribosomal protein (Var1); subunit 1 (Cox1), subunit 2 (Cox2), and subunit 3 (Cox3) of cytochrome oxidase; cytochrome *b* (Cytb); subunit 6 (Atp6), subunit 8 (Atp8) and subunit 9 (Atp9) of ATPase. Figure courtesy of Dr. Barrientos (Columbia University, New York City).

membrane insertion. The decreased amount of Cox2p in *cox16* and *cox15* mutants is therefore likely related to a general block in COX assembly. The reduction of newly synthesized Cox1p observed in the three mutants (*cox15*, *cox16*, and *cox18*) is a hallmark of most COX assembly mutants examined to date. HPLC analyses of mitochondrial hemes in both the C25/U1 and *cox16* null mutants revealed the presence of heme A and heme O (Figure 3.4) at levels comparable with other COX assembly mutants (Barros et al. 2001), suggesting that Cox16p is not involved in the synthesis of these molecules. Finally, the respiratory deficiency of C25/U1 and the *cox16* null mutant were not rescued by supplementation of the growth medium with copper, calcium, iron, magnesium, manganese, or zinc salts (Table 3.1). This result does not, however, exclude Cox16p from having a function in transport or insertion of one of the heavy metals known to be associated with COX.

COX16 encodes a 118 amino acid protein with a predicted mass of 14.1 kDa. The protein sequence is predicted to contain a single transmembrane domain comprised of amino acids 34-56, by the TMpred program (www.ch.embnet.org/software/TMPRED_form.html) (Figure 3.5). The presence of a mitochondrial targeting sequence at the amino-terminus (Figure 3.6) is predicted by the P-Sort program (//psort.nibb.ac.jp/helpwww2.html). The amino acid composition of Cox16p is biased toward acidic residues, and analysis of the putative mature protein reveals a pI of 5.0 (//ca.expasy.org/tools/pi_tool.html). Cox16p does not display any homology to proteins of known function in the most recent NCBI databases (August 2004) nor does it appear to have any identifiable functional domains that might provide clues about its function.

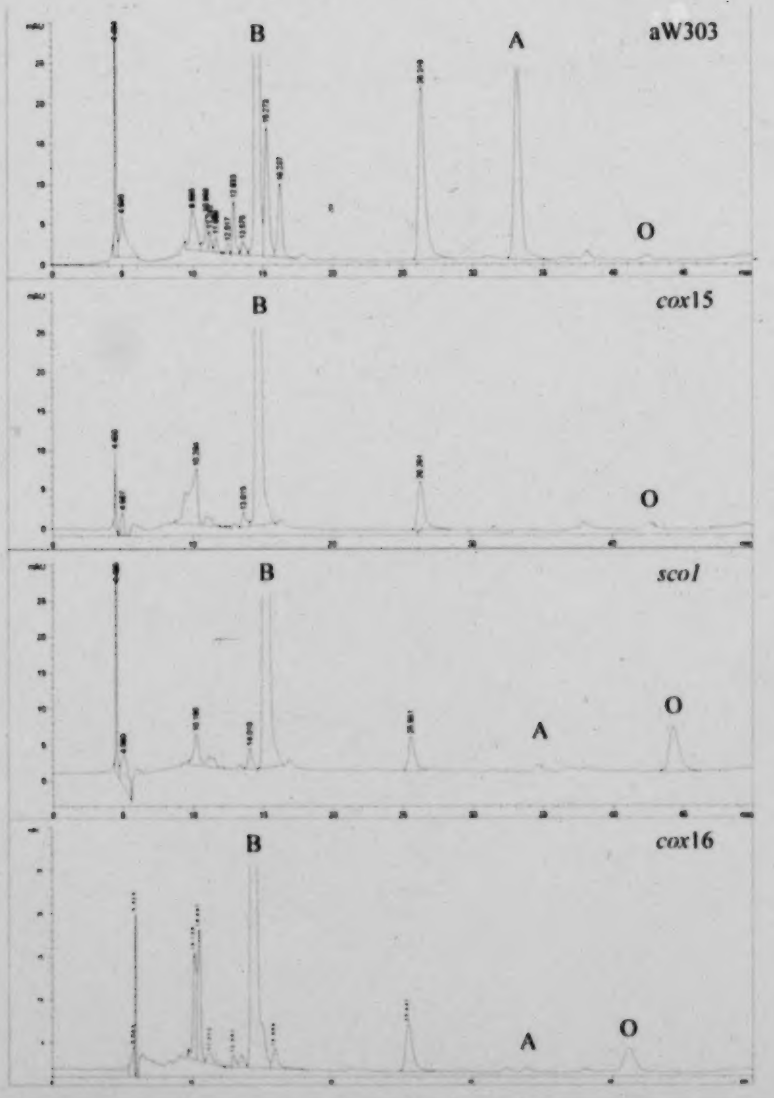

Figure 3.4. Analysis of mitochondrial hemes in wild type and mutant mitochondria. Mitochondria (3 mg) of the wild type strain W303-1A, aW303ΔCOX15 (*cox15* null mutant), and aW303ΔCOX16 (*cox16* null mutant) were extracted with acidic acetone and separated by reverse phase chromatography. The protoheme (B), heme O (O), and heme A (A) peaks were identified from the elution times of known standards and are marked above the appropriate peak.

Table 3.1. Metal Salt Suppression Analyses of a *cox16* null mutant

Strain	Ca	Cu	Fe	Mg	Mn	Zn
aW303	+O/N 0.01% 0.02% 0.05% 0.1% 0.2% 0.4% +O/2N 0.8%	+O/N 0.01% 0.02% 0.05% 0.1% +O/3N 0.2% -O/7N 0.4% 0.8%	+O/N 0.01% 0.02% 0.05% 0.1% +O/2N 0.2% -O/7N 0.4% 0.8%	+O/N 0.01% 0.02% 0.05% 0.1% 0.2% 0.4% 0.8%	+O/N 0.01% 0.02% 0.05% 0.1% 0.2% 0.4% -O/7N 0.8%	+O/N 0.01% 0.02% 0.05% 0.1% 0.2% -O/7N 0.4% 0.8%
aW303ΔCOX16	-O/7N All	-O/7N All	-O/7N All	-O/7N All	-O/7N All	-O/7N All
aW303ΔCOX17	-O/7N All	+O/4N 0.1% +O/7N 0.05% -O/7N 0.01% 0.02% 0.2% 0.4% 0.8%	-O/7N All	-O/7N All	-O/7N All	-O/7N All

All = at all concentrations; O/N = over one night; +/- = presence/absence of growth.

Concentrations (w/v) used were 0.01%, 0.02%, 0.05%, 0.1%, 0.2%, 0.4%, and 0.8%.

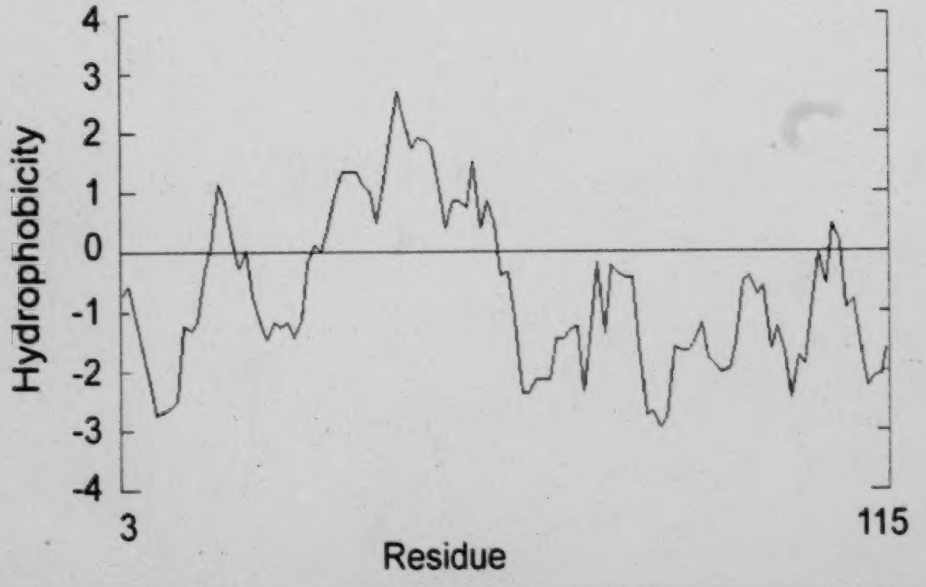

Figure 3.5. The *COX16* gene encodes a putative membrane protein. A hydropathy plot of Cox16p predicts a single membrane-spanning segment.

MSFSGKKFRSRRQQLVYEASLAGRYKKALSKHPFLFFGLPFCATIVLGSP

WLSSFTAIKYEQGDRKVQEINEEDILKIRKNQREFDIKEEYYRLQGLSEE

DWEPVRVARLKDESENVW

Figure 3.6. The *COX16* gene encodes a small, acidic protein. The primary amino acid sequence of Cox16p is shown with the potential mitochondrial targeting sequence denoted by the *bar above* the sequence. The predicted transmembrane domain is indicated by the *bar underneath* the sequence

Characterization of Cox16p

To determine if Cox16p was indeed a mitochondrial protein it was necessary to determine its subcellular location. As no antibody currently exists to Cox16p, localization was studied by generating a Myc epitope-tagged version of the protein. Since Cox16p is predicted to contain a cleavable amino-terminal signal sequence, the Myc epitope was added at the carboxy-terminus. Growth of transformants, expressing the Cox16p-Myc from either a high-copy episomal (aW303ΔCOX16/Myc1) or low-copy CEN plasmid (aW303ΔCOX16/Myc2), on non-fermentable carbon sources was indistinguishable from that of transformants expressing native Cox16p. Restoration of growth on these substrates correlated with the recovery of COX activity and respiratory competence. This was evident from both cytochrome aa_3 spectra (Figure 3.7) and Western analysis of COX subunits (Figure 3.2) in mitochondria purified from aW303Δ COX16/Myc1 and aW303ΔCOX16/Myc2. The concentrations of the three mitochondrially-encoded subunits, and Cox5p, in the transformants are similar to the wild-type levels, regardless of the vector used to express the Myc-tagged Cox16p (Figure 3.2). A low molecular weight protein is detected in mitochondria from aW303Δ COX16/Myc1 and aW303ΔCOX16/Myc2 when probed with a commercially available anti-Myc antibody (Figure 3.8). This band is absent in the PMS fraction from these strains and is not observed in wild type or aW303ΔCOX16 mitochondria. Based on its migration in a 12% SDS polyacrylamide gel, this protein has an apparent mass of 21 kDa, which is approximately 6 kDa larger than the combined size of native Cox16p and the additional 1 kDa contributed by the Myc epitope. The reason for the discrepancy, which may be even larger if Cox16p has a cleavable signal, is not clear but is probably related to

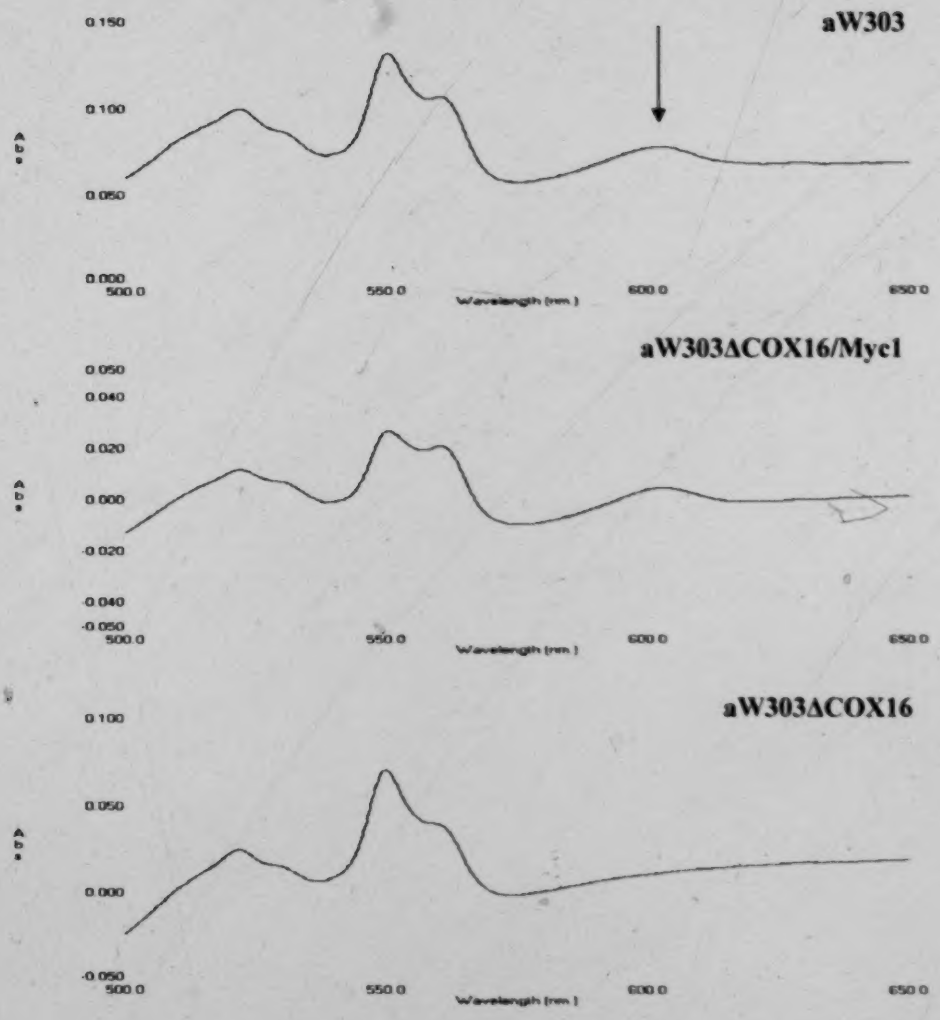

Figure 3.7. Spectral analysis of aW303ΔCOX16/Myc1 mitochondrial cytochromes. Mitochondria were prepared from the wild-type strain aW303, aW303ΔCOX16 and the strain aW303ΔCOX16/Myc1. The position of cytochrome aa_3 is marked.

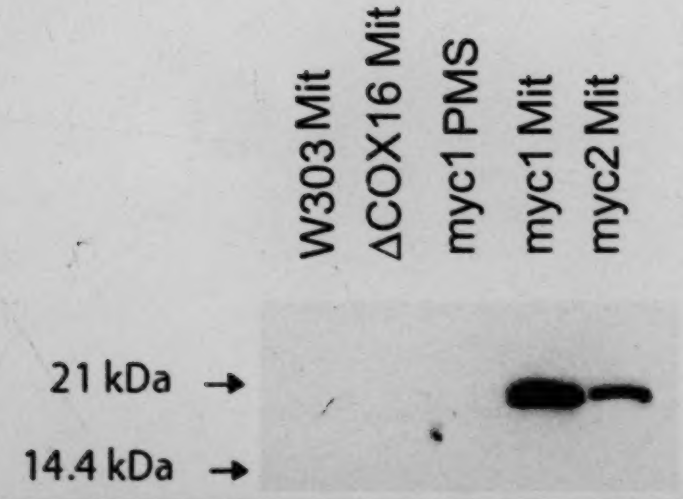

Figure 3.8. Cox16p localizes to the mitochondria. Western blot analysis of mitochondrial (Mit) fractions from the wild type strain W303-1A (W303), the *cox16* mutant aW303ΔCOX16 (ΔCOX16), and the transformants aW303Δ COX16/Myc1 (Myc1) and aW303ΔCOX16/Myc2 (Myc2), expressing a Myc-tagged Cox16p from a high copy plasmid and from a CEN plasmid, respectively. The post-mitochondrial fractions (PMS) from the strain over-expressing Cox16-Myc were also analyzed. Mitochondrial (20 μg) or postmitochondrial supernatant fractions (40 μg) were separated in 12% polyacrylamide gels and transferred to nitrocellulose, and the blot probed with a monoclonal antibody specific to the Myc epitope tag.

anomalous binding of SDS by this acidic protein. Almost all of the COX assembly factors identified to date are constituents of the IMM, as would be expected from their involvement in assembly of a hydrophobic multimeric complex of this membrane. The prediction of a single transmembrane domain in Cox16p suggested that it might be an integral membrane protein. This was confirmed by testing the solubility properties of the protein. Titration of aW303ΔCOX16/Myc1 mitochondria with increasing concentrations of deoxycholate, in the presence of 0.5 M NaCl, demonstrate that extraction of Cox16p-Myc requires a minimum of 0.25% detergent, which is assessed by our ability to detect Cox16p in the supernatant (Figure 3.9). Results for the Myc2, which expresses Cox16p from a low copy plasmid were identical to that of Myc1.

In order to determine the submitochondrial localization of Cox16p, proteinase K protection assays in intact mitochondria were performed. Western blot analysis of intact mitochondria and mitoplasts from aW303ΔCOX16/Myc1 demonstrate that Cox16p is a constituent of the IMM, as it is only found in the pelleted, mitoplast fraction (Figure 3.10). Treatment with proteinase K results in Cox16p being degraded in mitoplasts, but not in intact mitochondria. The decreased signal observed in the proteinase K-treated mitochondrial fraction is probably due to a subpopulation of mitochondria with damaged outer membranes, which would expose the otherwise protected Cox16p. Similar results were obtained when mitochondria and mitoplasts were probed with antibody against Sco1p, a IMM protein with a carboxy- terminus known to protrude into the IMS (Beers et al. 1997). Under the same conditions, Cox5p is protected from proteinase K digestion in mitoplasts. Cox5p is also an IMM protein, but in contrast to Sco1p, it has minimal protrusion into the IMS.

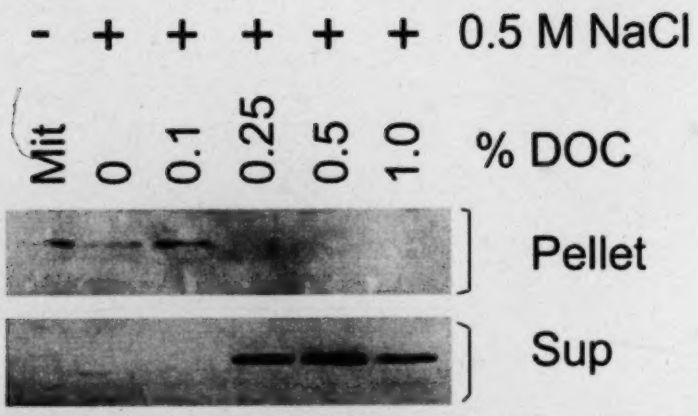

Figure 3.9. Cox16p is an integral membrane protein. Mitochondria from aW303ΔCOX16/Myc1 were extracted in the absence (–) or presence (+) of 0.5 M NaCl and increasing concentrations of deoxycholate (*DOC*). Following centrifugation at 100,000 × *g*, the pellet (containing mitochondrial membrane with non-extracted protein) and supernatant (containing proteins extracted from the mitochondrial membrane) fractions were normalized back to the starting volume of mitochondria and separated on a 15% gel and analyzed as described in Figure 3.8.

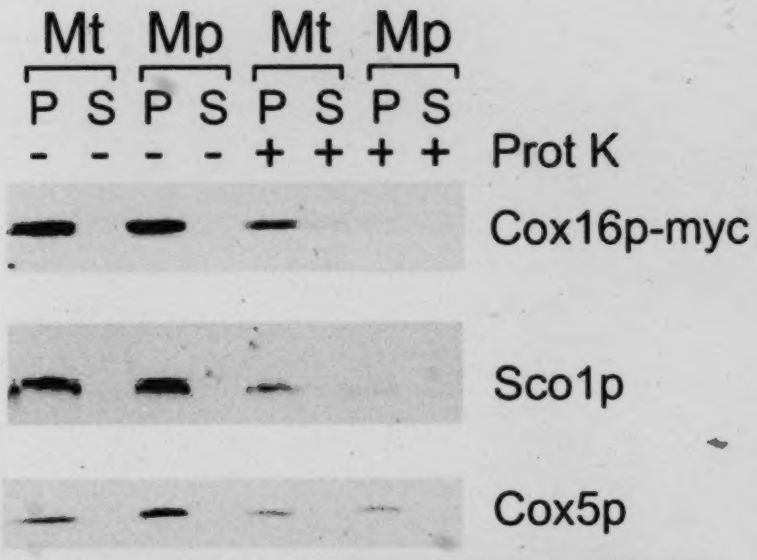

Figure 3.10. Cox16p is an IMM protein. Mitochondria with intact outer membranes were isolated and converted to mitoplasts by the method of Glick and Pon (Glick 1995). The equivalent of 1 mg of mitochondrial protein starting material was incubated in the presence or absence of 16 μg of proteinase K for 30 minutes, and each fraction analyzed for the presence of the Myc-tagged Cox16p, Sco1p, or Cox5p.
MT = mitochondria; MP = mitoplasts; p = pellet; S = supernatant; PK = proteinase K; + or − = presence or absence of proteinase K.

Several COX assembly factors characterized thus far appear to exist natively as part of higher molecular weight homo- and/or heteroligomeric complexes, even though none of them are associated with the final assembled COX holoenzyme (Glerum et al. 1995; Lode et al. 2000; Heaton et al. 2001; Barrientos et al. 2002). The native molecular mass of Cox16p-Myc was estimated to be ~84 kDa for both aW303ΔCOX16/Myc1 and aW303Δ COX16/Myc2 (Figure 3.11). This was determined by sedimentation of a 1% deoxycholate extract of mitochondria, along with the internal controls LDH and Hb, in a 7 to 20% linear sucrose gradient.

Cox16p Has Mammalian Homologs

Searches of protein and EST databases indicate that Cox16p is conserved in the yeast, *S. pombe* and appears to have human (HSCP203; accession number NP_057552) and murine (accession number NP_079737) homologues. The human cDNA was originally identified in a screen for novel proteins expressed in CD34+ hematopoietic stem/progenitor cells (Zhang et al. 2000). Human *COX16* is located on the long arm of chromosome 14, in the interval 14q24.1 (LOC51241) (http://www.ncbi.nlm.nih.gov/). Figure 3.12 presents an alignment of the Cox16 proteins from two species of yeast (*S. cerevisiae* and *S. pombe*), humans, and one species of mouse. This analysis reveals that the highest sequence conservation is in the region of the transmembrane domain and the carboxy-terminal half of the protein. The four Cox16 proteins shown in Figure 3.12 share 24% identity and 40% conserved residues. To test whether the HSPC203 cDNA could functionally complement the yeast *cox16* null mutant, HSPC203 was amplified by PCR from a HeLa cell cDNA library. The cDNA was cloned into a yeast expression vector

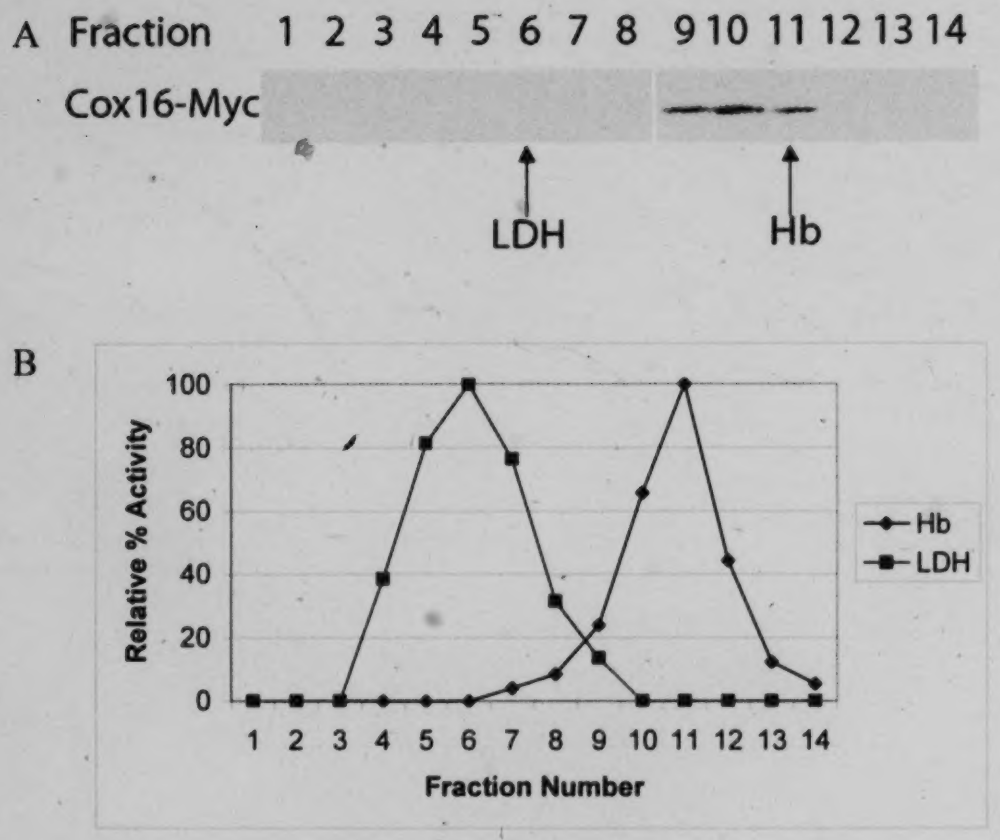

Figure 3.11. Cox16-Myc is found in a higher molecular weight complex. 7-20% sucrose gradients were loaded with samples containing protein from extracted Myc1 mitochondria. (A) 25 µl of each fraction was used for Western blot analysis of Cox16-Myc1 (as described in Fig. 3.2). (B) Shows a graphic representation of internal standards. Hb (67 kDa) absorbance was measured at 410 nm and LDH (130 kDa) activity was measured at 340nm.

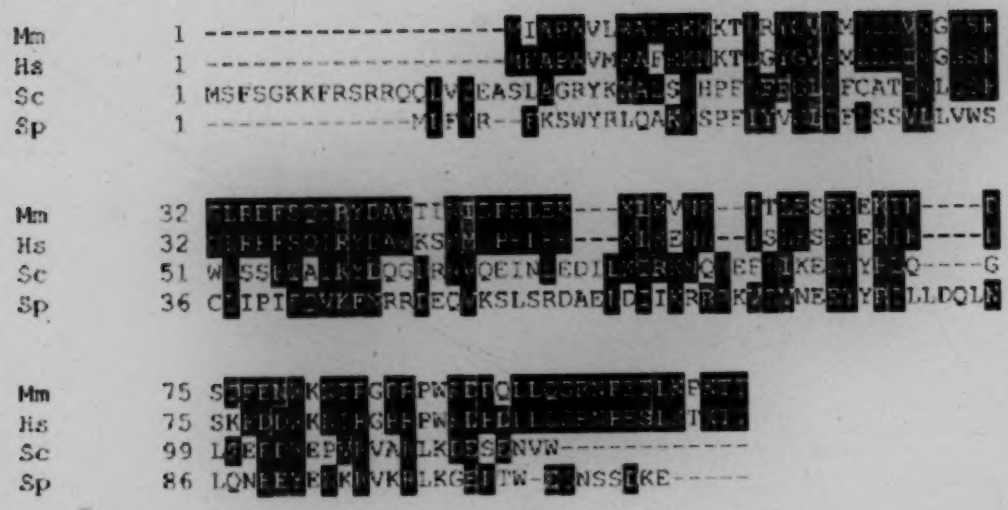

Figure 3.12. Cox16p has murine and human homologues. An alignment of *S. cerevisiae* (Sc) and *S. pombe* (Sp) Cox16ps with the murine (Mm) and human (Hs) Cox16 homologs. The alignment was generated by the ClustalW program (//www.ebi.ac.uk/clustalw/) and shaded with the Boxshade Program (//www.ch.embnet.org/software/BOX_form.html). Identical residues are shaded in *black*, and conservative replacements are shaded in *gray*.

(pMGL3) containing the *ADH1* promoter and terminator in a YEp351 backbone, allowing constitutive expression of proteins from cDNAs with their own start codons. This construct (pCOX16H/ST1) did not complement the respiratory deficiency of the aW303Δ COX16 parent strain even after incubation on a non-fermentable carbon source for 7 days, whereas the yeast *COX16* gene restores complete respiratory competence and grows after one night on EG (Figure 3.13). These results indicate that while the human gene is homologous, it is not orthologous, to yeast *COX16*.

Towards Identifying a Function for Cox16p

As a first step in elucidating a specific function for Cox16p, we wanted to identify the important domains of yeast Cox16p and a site-directed mutagenesis approach was undertaken. Cox16p homologs were aligned using ClustalW and highly conserved residues were identified. A total of 18 residues in the transmembrane domain and carboxy-terminus were mutated (Figure 3.14), using the low-copy plasmid pCox16/St5 as a template. Mutations were confirmed by capillary sequencing with the AB 3100 Avant capillary sequencer (Applied Biosystems) and mutant constructs transformed into aW303ΔCOX16. Of these 18 mutants, 8 demonstrated an abnormal growth phenotype when incubated on the non-fermentable carbon source EG (Table 3.2). Two strains harboring the mutations R83E and Y91D, respectively, required three days to grow, compared to the wild-type aW303. Six other mutants (V46F, L47F, E90K, R93D, R106D, V107F) grew over 2 nights, suggesting mild dysfunction of Cox16p in these strains. The remaining 10 strains grew overnight, suggesting that these mutations did not have any affect on Cox16p function.

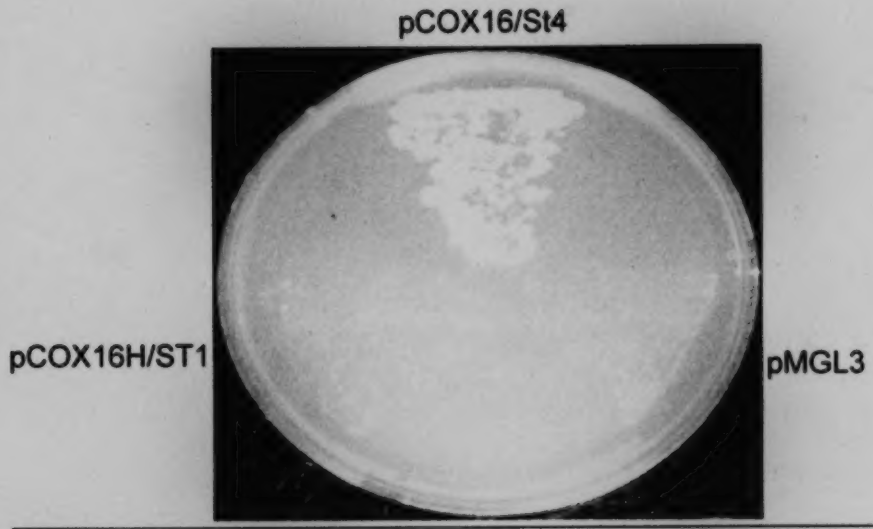

Figure 3.13. h*COX16* does not complement a Sc*cox16* null mutant. Human *COX16* (pCOX16H/ST1), Sc*COX16* (pCOX16/St4), and the negative control plasmid, pMGL3, were transformed into aW303ΔCOX16 and grown on EG media. No growth was observed for either pCOX16H/ST1 or pMGL3 after 7 days.

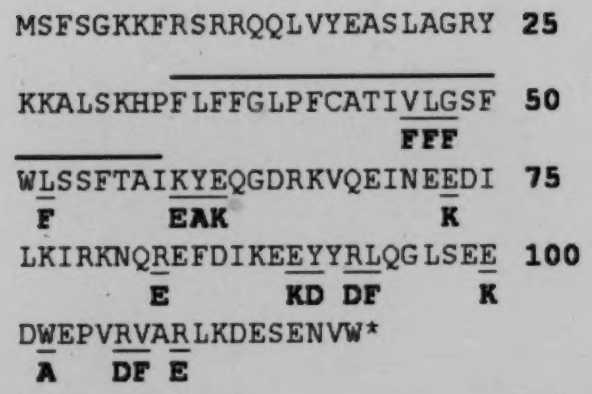

Figure 3.14. The amino acid sequence of Cox16p showing mutations introduced by site-directed mutagenesis. Mutated amino acids are *underlined* and mutations are shown in bold. The predicted transmembrane domain is indicated by the *bar above* the sequence.

Table 3.2. Growth rates of *cox16* point-mutant strains on EG.[1]

Mutation	Growth on EG
aW303 (wild-type)	+O/N
V46F	+O/2N
L47F	+O/2N
R83E	+O/3N
E90K	+O/2N
Y91D	+O/3N
R93D	+O/2N
R106D	+O/2N
V107F	+O/2N

1 – Listed growth rates only include those mutants that had an abnormal growth rate. The remaining site-directed mutants grew overnight, comparable to wild type yeast.

Chapter 4: Discussion

On the Characterization of Cox16p

The biogenesis of COX is a complex process requiring the coordinated interaction of gene products from two distinct genomes, the mitochondrial and the nuclear. Assembly of COX requires not only the structural polypeptides of the complex, but also a host of nuclear-encoded ancillary factors, some of which are required to synthesize the prosthetic groups. Thus far, over 30 assembly factors have been identified in yeast. While we have gained an incredible amount of information about the structure of COX, the mechanism by which this multimeric complex assembles remains unclear. Mutations in any requisite COX assembly factor result in a specific COX deficiency and loss of respiratory competence. Mutations in six COX assembly factors (COX10, COX15, LRPPRC, SCO1, SCO2, SURF1) have now been shown to cause human COX deficiencies. Loss of these proteins results in a broad and heterogeneous range of clinical phenotypes, with allelic heterogeneity in some of the genes being reflected by signs and symptoms exhibited by the patients. It is unclear why these proteins, which are ubiquitously expressed at varying levels, result in tissue-specific disease. Delineating the tissue-specific assembly and regulation of COX will be the subject of many future studies.

Unfortunately, for the vast majority of COX deficient patients, the underlying molecular basis of the disease is unknown. As no mutations have been observed in any of the nuclear-encoded structural proteins in these cases, it is presumed that most of these COX-deficiencies arise from mutations in assembly factors. With the exception of SURF1, all the known human COX assembly factors have been identified using a candidate gene approach based on previously identified homologues in yeast. The yeast

S. cerevisiae is an ideal model system for the study of COX biogenesis. It is a facultative anaerobe, which allows mutants without a functioning ETC to grow on fermentable carbon sources. It is also very amenable to biochemical and genetic studies and has been demonstrated to be a powerful tool for the identification and characterization of COX assembly proteins. Many of these proteins are highly conserved between yeast and humans, which enables characterization in yeast to facilitate studies of the human COX homologues. One such assembly factor, Cox16p, is the focus of this thesis and my characterization of this novel yeast protein has been described in Chapter 3.

COX16 is a nuclear gene that was originally identified by its ability to restore respiratory competence to mutants of complementation group G22 (Tzagoloff and Dieckmann 1990), which are specifically deficient in COX. Since Cox16p is not part of the final assembled COX complex (Tsukihara and Yoshikawa 1998), it is assumed to have a role in the assembly of this multimeric complex. *COX16* encodes a small protein with a predicted mass of 14.1 kDa. With the addition of the Myc-epitope, the mass of the protein would be approximately 15 kDa. Cox16p-Myc, however, appears to migrate as an approximately 21 kDa protein on SDS-PAGE. This may be a consequence of the mature Cox16p being an acidic protein with a pI of 5.0. The negative charge could affect the uniform binding of SDS, causing aberrant migration in the gel. The Myc-epitope is also acidic and may exaggerate this effect. Transformation of *cox16* cells with either a high copy episomal (Myc1) or a low copy centromeric plasmid (Myc2) containing a *COX16-MYC* fusion gene was capable of restoring respiratory competence, as well as the comparable cytochrome aa_3 spectral signal, indicative of COX being correctly assembled. These data suggest that the function of Cox16p is not compromised by the

presence of the Myc-epitope and that Cox16p-Myc can be considered functionally equivalent to wild-type Cox16p. As observed in other COX assembly mutants, the steady-state concentrations of Cox1p, Cox2p, and Cox3p appear markedly decreased in the *cox16* null mutant. The steady-state levels of the nuclear-encoded subunits are normal, with the exception of Cox5p, whose expression is slightly decreased compared with wild-type. The significance of the loss of Cox5p is not clear and the relevance of this observation to the function of Cox16p is unknown. It may be that the decreased detection of Cox5p is specifically a consequence of the absence of Cox16p and that this protein is necessary to maintain adequate levels of Cox5p.

In an effort to determine whether Cox16p may have a role in heme A biosynthesis, HPLC analyses were performed. Proteins known to be involved in the heme A biosynthetic pathway, such as Cox10p and Cox15p, demonstrate abnormally low or absent heme O and/or heme A levels. Extracts from *cox16* mitochondria demonstrate heme O and heme A levels comparable to other COX assembly mutants, so it does not appear that Cox16p is involved in heme metabolism or heme A insertion into Cox1p.

As COX is a metalloprotein with numerous prosthetic groups, I wanted to examine if Cox16p had a function in the maturation of any of the metal centers. While the formation of the heme A and copper metal sites have been well studied, our knowledge of these pathways remains incomplete. As well, there have been no reported studies on the mechanisms by which the non-redox active metal centers (Mg, Na and Zn) are formed. The respiratory deficiency of *cox16* is not rescued by supplementation of the growth media with copper, calcium, iron, magnesium, manganese, or zinc salts. These results suggest, but do not exclude the possibility, that Cox16p does not have a role in

transport or insertion of these heavy metals for maturation of the COX metal centers. Analogous to this, mutations in Sco1p, a protein known to bind copper and proposed to transfer copper to the Cu_A site of Cox2p, are not rescued by the presence of exogenous copper in the media. In contrast, over-expression of Cox17p and the addition of exogenous copper are capable of rescuing a *sco2* mutant, which is another protein thought to be involved in the same pathway. It would be interesting to observe whether over-expression of known chaperones in the various metal pathways, combined with the addition of an appropriate exogenous metal to the growth media, would be capable of suppressing the *cox16* COX deficiency.

The Cox16p-Myc fusion protein allowed me to demonstrate that Cox16p localizes strictly to the mitochondria. It did not have a dual localization such as that observed with Cox17p, Cox19p or Cox23p. Extraction of Cox16p from mitochondrial membranes required a minimum of 0.25% deoxycholate, which is characteristic of most of the other COX assembly proteins that are integral membrane components of the IMM. As mitochondria contain two membranes, I wanted to determine whether Cox16p localizes to the outer or inner membrane. Treatment of mitochondria and mitoplasts with proteinase K resulted in the loss of a detectable signal only in the mitoplast fraction, suggesting that Cox16p is a component of the IMM, which is consistent with its proposed role as a COX assembly factor. The absence of Cox16p-Myc signal in the mitoplast sample after treatment with proteinase K suggests that the carboxy-terminus of this protein is located in the IMS. Had the carboxy-terminus been present in the matrix, there would have been a detectable signal in the proteinase K-treated mitoplasts, since it would have been inaccessible to cleavage. Combined with the TMpred prediction of a single

transmembrane domain, it suggests that Cox16p has an amino-in, carboxy-out orientation in the membrane (Figure 4.1). The orientation of Cox16p, especially if the amino-terminal contains a cleavable mitochondrial targeting signal (as predicted by the PSORT program), would suggest that the active site of this protein is found at its carboxy-terminus in the IMS. Whether Cox16p actually does contain an amino-terminal targeting signal has not been determined, and the mechanism by which this protein inserts into the IMM is not known. Presumably, the insertion of Cox16p will occur in one of two ways: (1) after recognition and transport by the TOM complex, Cox16p is transported across the IMS and inserted directly into the IMM by the TIM22 complex; or (2) Cox16p is translocated by TOM across the outer membrane, transferred directly to the TIM23 complex in the IMM, and a stop-transfer activity is exerted by its transmembrane domain, which halts translocation, so it moves laterally into the membrane. In either case, the amino-terminal signal sequence is then cleaved off in the mitochondrial matrix. I feel that the second option is more likely, as proteins inserted in this manner generally contain an amino-terminal, cleavable localization signal. The first pathway typically involves uncleaved, internal signal motifs that target proteins to the IMM. It also seems unlikely that Cox16p gets inserted via the matrix by the Oxa1p IMM insertion pathway, as all proteins known to be inserted by this mechanism have an amino-out (in the IMS)/carboxy-in (matrix) orientation in the IMM, which I have demonstrated that Cox16p does not have .

Based on sucrose density gradient analysis, native Cox16p appears to be found in an oligomeric complex slightly larger than hemoglobin (67 kDa), but smaller than LDH (130 kDa). Several other assembly factors are known to exist in either hetero-oligomeric

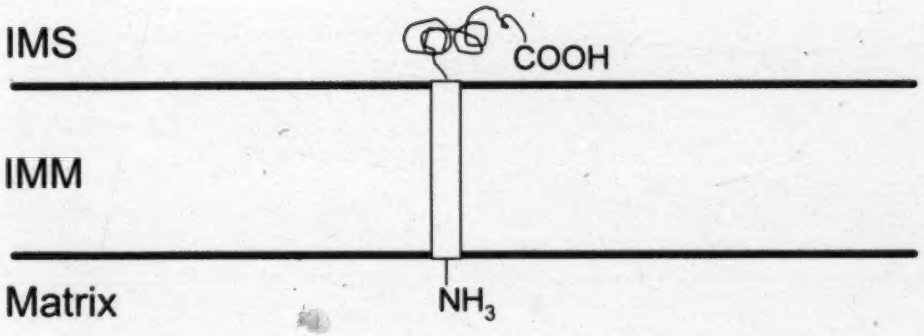

Figure 4.1. Schematic diagram of Cox16p orientation in the IMM.

or homo-oligomeric complexes within the mitochondria. Determination of the composition of the Cox16p complex could provide insight into the function of this protein. These studies could be accomplished by Co-IP and cross-linking experiments to determine whether Cox16p exists as a hetero- or homo-oligomer. Co-transformation of the aW303ΔCOX16 strain with Flag- and Myc-epitope tagged Cox16p will allow pull-down assays to be performed, using commercially available columns that are coated with anti-Flag antibodies. Following separation by SDS-PAGE, Western blotting with anti-Myc antibody would help determine whether Cox16p binds itself in a homo-oligomer. Alternatively, silver staining of the SDS-PAGE, followed by mass spectrometry would allow identification of protein-binding partners if Cox16p exists in a hetero-oligomer. The possibility exists that the migration of the two soluble internal standards, Hb and LDH, were affected by the presence of the deoxycholate detergent, which could have also affected the Cox16p-Myc migration. This could be tested by Western blot analysis of other membrane proteins, such as Sco1p, for which the size of the complex is known.

BLAST database analyses demonstrate that COX16 is very conserved in fungi, but that it also has homologues in the murine and human genomes. Transformation of human *COX16* into aW303ΔCOX16 mutant is not capable of functionally complementing and the null mutant remains respiratory deficient. This may indicate that human COX16 has a different function than Cox16p, or that the stability/function of this protein is affected in the heterologous yeast environment. The inability of human COX16 to functionally complement its yeast counterpart is not unique among COX assembly factors, as only about one-third of human COX assembly factors identified to date have been found to be capable of rescuing null alleles of their yeast counterparts.

In silico studies have not offered insight into Cox16p function, as this protein, along with its homologues, does not contain any motifs that may indicate a role in any of the described COX assembly pathways. This may suggest that Cox16p is involved in a unique pathway not yet reported. To address this problem, and identify the functional domains of Cox16p, I generated a series of specific *cox16* point mutants. Residues were chosen to be mutated based on their conservation between higher and lower eukaryotes (as determined by BLAST sequence queries and CLUSTALW alignments). Eight mutants demonstrated an abnormal respiratory phenotype on EG. Of these, two mutants (both of which had mutations in the carboxy-terminus) required 3 days to reach a colony density equal to one night of wild-type growth, while the remaining six mutants required +/- 2 nights. These mutants now need to be characterized biochemically, using the standard techniques described in Chapters 2 and 3. All of the mutants will also need to be screened (using techniques such as sucrose gradient density centrifugation and gel filtration) for their ability to oligomerize. These studies could also be extended to Co-IP and cross-linking experiments, to determine whether the mutant Cox16p proteins still exist in a homo- or hetero-oligomer state or whether specific binding sites are affected by the mutations. Cotransformation of the yeast strain aW303Δ*COX16* with Flag- and Myc-epitope tagged Cox16p will allow pulldown assays to be performed to determine if Cox16p oligomer formation is affected.

Based on a suitable phenotype, which would include stable expression of Cox16p, combined with an inability to grow on EG and an abnormal spectral absorbance at 603nm, a subset of mutants could be further studied in multicopy suppressor and synthetic lethal genetic screens. These are powerful yeast genetic tools for identifying

protein-protein interactions, as well as other genetic interactions, and frequently allow determination of specific protein functions. Suppressors identified in these screens could be characterized both genetically and biochemically, which will ultimately allow us to decipher a specific function for Cox16p in the COX assembly pathway.

As we are also interested in understanding the molecular basis for human COX deficiencies, it is important that the h*COX16* gene be screened for mutations in our growing collection of COX-deficient patients, since the underlying molecular cause for the defects remains unknown. In addition, it is necessary to begin characterization of h*COX16* (HSPC203) to determine if the protein does indeed localize to the IMM in humans. Our lab has also recently determined that h*COX16* actually expresses two mRNA splice variants, one of which contains the complete *COX16* sequence and the other of which is missing most of the transmembrane domain (amino acids 34-56). Presumably, if the transmembrane domain of COX16 is missing, it would also be missing its stop/transfer signal, which would normally cause retention of the protein in the IMM. Loss of this signal sequence might result in COX16 import into the mitochondrial matrix or IMS rather than the IMM. As COX16 is predicted to have its functional domain at its carboxy-terminus in the IMS, it would be intriguing to identify any potentially new function for this protein. To explore this further, I began to generate multiple constructs for each splice product with a FLAG-epitope tag added to the amino- or carboxy-terminus. Transfection of the h*COX16-FLAG* fusion gene into a mammalian cell line, followed by the use of a fluorescently-labelled anti-FLAG antibody, will allow visualization of COX16's localization by confocal microscopy. These experiments should provide further insight into Cox16p oligomerization and function.

The characterization of Cox16p remains in the early stages, with much work to be done. Through the biochemical study of *cox16* point mutants, identification of Cox16p binding partners in the oligomeric complex, and characterization of h*COX16* in mammalian cells, the combined information from these studies will help elucidate a function for Cox16p and the nature of its role in the COX assembly pathway. The ultimate goal of these studies is to utilize the knowledge gained from studies in yeast to understand the function of the COX16 protein in humans and to determine whether/how this unique protein may play a role in the etiology of inherited human COX deficiencies. As the underlying molecular cause of most COX deficiencies remains a mystery, the identification and characterization of proteins involved in the assembly of cytochrome oxidase will offer potential therapeutic targets for treatment of these lethal human diseases.

Bibliography:

Agostino, A., F. Invernizzi, C. Tiveron, G. Fagiolari, A. Prelle, E. Lamantea, A. Giavazzi, G. Battaglia, L. Tatangelo, V. Tiranti and M. Zeviani (2003). "Constitutive knockout of *Surf1* is associated with high embryonic lethality, mitochondrial disease and cytochrome *c* oxidase deficiency in mice." Hum Mol Genet **12**(4): 399-413.

Amaravadi, R., D. M. Glerum and A. Tzagoloff (1997). "Isolation of a cDNA encoding the human homolog of *COX17*, a yeast gene essential for mitochondrial copper recruitment." Hum Genet **99**(3): 329-33.

Anderson, S., A. T. Bankier, B. G. Barrell, M. H. de Bruijn, A. R. Coulson, J. Drouin, I. C. Eperon, D. P. Nierlich, B. A. Roe, F. Sanger, P. H. Schreier, A. J. Smith, R. Staden and I. G. Young (1981). "Sequence and organization of the human mitochondrial genome." Nature **290**(5806): 457-65.

Antonicka, H., S. C. Leary, G. H. Guercin, J. N. Agar, R. Horvath, N. G. Kennaway, C. O. Harding, M. Jaksch and E. A. Shoubridge (2003). "Mutations in *COX10* result in a defect in mitochondrial heme A biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency." Hum Mol Genet **12**(20): 2693-702.

Antonicka, H., A. Mattman, C. G. Carlson, D. M. Glerum, K. C. Hoffbuhr, S. C. Leary, N. G. Kennaway and E. A. Shoubridge (2003). "Mutations in *COX15* produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy." Am J Hum Genet **72**(1): 101-14.

Balatri, E., L. Banci, I. Bertini, F. Cantini and S. Ciofi-Baffoni (2003). "Solution structure of Sco1: a thioredoxin-like protein involved in cytochrome *c* oxidase assembly." Structure (Camb) **11**(11): 1431-43.

Barrientos, A., M. H. Barros, I. Valnot, A. Rotig, P. Rustin and A. Tzagoloff (2002). "Cytochrome oxidase in health and disease." Gene **286**(1): 53-63.

Barrientos, A., D. Korr and A. Tzagoloff (2002). "Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of Leigh's syndrome." Embo J **21**(1-2): 43-52.

Barros, M. H., C. G. Carlson, D. M. Glerum and A. Tzagoloff (2001). "Involvement of mitochondrial ferredoxin and Cox15p in hydroxylation of heme O." FEBS Lett **492**(1-2): 133-8.

Barros, M. H., A. Johnson and A. Tzagoloff (2004). "*COX23*, a homologue of *COX17*, is required for cytochrome oxidase assembly." J Biol Chem **279**(30): 31943-7.

Barros, M. H. and F. G. Nobrega (1999). "*YAH1* of *Saccharomyces cerevisiae*: a new essential gene that codes for a protein homologous to human adrenodoxin." Gene **233**(1-2): 197-203.

Barros, M. H., F. G. Nobrega and A. Tzagoloff (2002). "Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*." J Biol Chem **277**(12): 9997-10002.

Barros, M. H. and A. Tzagoloff (2002). "Regulation of the heme A biosynthetic pathway in Saccharomyces cerevisiae." FEBS Lett **516**(1-3): 119-23.

Beers, J., D. M. Glerum and A. Tzagoloff (1997). "Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle." J Biol Chem **272**(52): 33191-6.

Beers, J., D. M. Glerum and A. Tzagoloff (2002). "Purification and characterization of yeast Sco1p, a mitochondrial copper protein." J Biol Chem **277**(25): 22185-90.

Berg, J. T., John L.; Stryer, Lubert (2002). Biochemistry. New York, W.H. Freeman and Company.

Bernhardt, G. A. (1995). Cytochrome P450: Structure, function and generation of reactive oxygen species. Rev Physiol Biochem Pharmacol. M. Grunicke, Springer-Verlag: 137-221.

Broadley, S. A., C. M. Demlow and T. D. Fox (2001). "Peripheral mitochondrial inner membrane protein, Mss2p, required for export of the mitochondrially coded Cox2p C tail in Saccharomyces cerevisiae." Mol Cell Biol **21**(22): 7663-72.

Brown, B. M., Z. Wang, K. R. Brown, J. A. Cricco and E. L. Hegg (2004). "Heme O Synthase and Heme A Synthase from *Bacillus subtilis* and *Rhodobacter sphaeroides* Interact in *Escherichia coli*." Biochemistry **43**(42): 13541-8.

Brown, K. R., B. M. Allan, P. Do and E. L. Hegg (2002). "Identification of novel hemes generated by heme A synthase: evidence for two successive monooxygenase reactions." Biochemistry **41**(36): 10906-13.

Bruno, C., A. Martinuzzi, Y. Tang, A. L. Andreu, F. Pallotti, E. Bonilla, S. Shanske, J. Fu, C. M. Sue, C. Angelini, S. DiMauro and G. Manfredi (1999). "A stop-codon mutation in the human mtDNA cytochrome *c* oxidase I gene disrupts the functional structure of complex IV." Am J Hum Genet **65**(3): 611-20.

Buchwald, P., G. Krummeck and G. Rodel (1991). "Immunological identification of yeast SCO1 protein as a component of the inner mitochondrial membrane." Mol Gen Genet **229**(3): 413-20.

Capaldi, R. A. (1990). "Structure and assembly of cytochrome c oxidase." Arch Biochem Biophys **280**(2): 252-62.

Carlson, C. G., A. Barrientos, A. Tzagoloff and D. M. Glerum (2003). "*COX16* encodes a novel protein required for the assembly of cytochrome oxidase in Saccharomyces cerevisiae." J Biol Chem **278**(6): 3770-5.

Carr, H. S., G. N. George and D. R. Winge (2002). "Yeast Cox11, a protein essential for cytochrome c oxidase assembly, is a Cu(I)-binding protein." J Biol Chem **277**(34): 31237-42.

Caughey, W. S., G. A. Smythe, D. H. O'Keeffe, J. E. Maskasky and M. I. Smith (1975). "Heme A of cytochrome *c* oxicase. Structure and properties: comparisons with hemes B, C, and S and derivatives." J Biol Chem **250**(19): 7602-22.

Chen, Z. W. (1997). "Porcine Cox17p/dopuin possesses high sequence homology to the product of human *COX17* gene (h-Cox17p)." Eur. J. Biochem **249**: 518-522.

Chinenov, Y. V. (2000). "Cytochrome *c* oxidase assembly factors with a thioredoxin fold are conserved among prokaryotes and eukaryotes." J Mol Med **78**(5): 239-42.

Chinnery, P. F. and E. A. Schon (2003). "Mitochondria." J Neurol Neurosurg Psychiatry **74**(9): 1188-99.

Clark, K. M., R. W. Taylor, M. A. Johnson, P. F. Chinnery, Z. M. Chrzanowska-Lightowlers, R. M. Andrews, I. P. Nelson, N. W. Wood, P. J. Lamont, M. G. Hanna, R. N. Lightowlers and D. M. Turnbull (1999). "An mtDNA mutation in the initiation codon of the cytochrome C oxidase subunit II gene results in lower levels of the protein and a mitochondrial encephalomyopathy." Am J Hum Genet **64**(5): 1330-9.

Coenen, M. J., L. P. van den Heuvel, L. G. Nijtmans, E. Morava, I. Marquardt, H. J. Girschick, F. J. Trijbels, L. A. Grivell and J. A. Smeitink (1999). "*SURFEIT-1* gene analysis and two-dimensional blue native gel electrophoresis in cytochrome *c* oxidase deficiency." Biochem Biophys Res Commun **265**(2): 339-44.

Cole, E. G. and K. Gaston (1997). "A functional YY1 binding site is necessary and sufficient to activate *Surf-1* promoter activity in response to serum growth factors." Nucleic Acids Res **25**(18): 3705-11.

Comi, G. P., A. Bordoni, S. Salani, L. Franceschina, M. Sciacco, A. Prelle, F. Fortunato, M. Zeviani, L. Napoli, N. Bresolin, M. Moggio, C. D. Ausenda, J. W. Taanman and G. Scarlato (1998). "Cytochrome *c* oxidase subunit I microdeletion in a patient with motor neuron disease." Ann Neurol **43**(1): 110-6.

Coruzzi, G. and A. Tzagoloff (1979). "Assembly of the mitochondrial membrane system. DNA sequence of subunit 2 of yeast cytochrome oxidase." J Biol Chem **254**(18): 9324-30.

Dahl, H. H. and D. R. Thorburn (2001). "Mitochondrial diseases: beyond the magic circle." Am J Med Genet **106**(1): 1-3.

Dickinson, E. K., D. L. Adams, E. A. Schon and D. M. Glerum (2000). "A human *SCO2* mutation helps define the role of Sco1p in the cytochrome oxidase assembly pathway." J Biol Chem **275**(35): 26780-5.

DiMauro, S. and E. A. Schon (2001). "Mitochondrial DNA mutations in human disease." Am J Med Genet **106**(1): 18-26.

Field, L. S., E. Luk and V. C. Culotta (2002). "Copper chaperones: personal escorts for metal ions." J Bioenerg Biomembr **34**(5): 373-9.

Gattermann, N., S. Retzlaff, Y. L. Wang, G. Hofhaus, J. Heinisch, C. Aul and W. Schneider (1997). "Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome c oxidase in two patients with acquired idiopathic sideroblastic anemia." Blood **90**(12): 4961-72.

Gietz, R. D. and A. Sugino (1988). "New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites." Gene **74**(2): 527-34.

Glerum, D. M., T. J. Koerner and A. Tzagoloff (1995). "Cloning and characterization of *COX14*, whose product is required for assembly of yeast cytochrome oxidase." J Biol Chem **270**(26): 15585-90.

Glerum, D. M., I. Muroff, C. Jin and A. Tzagoloff (1997). "*COX15* codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase." J Biol Chem **272**(30): 19088-94.

Glerum, D. M., A. Shtanko and A. Tzagoloff (1996). "Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase." J Biol Chem **271**(24): 14504-9.

Glerum, D. M., A. Shtanko and A. Tzagoloff (1996). "*SCO1* and *SCO2* act as high copy suppressors of a mitochondrial copper recruitment defect in Saccharomyces cerevisiae." J Biol Chem **271**(34): 20531-5.

Glerum, D. M. and A. Tzagoloff (1994). "Isolation of a human cDNA for heme A:farnesyltransferase by functional complementation of a yeast cox10 mutant." Proc Natl Acad Sci U S A **91**(18): 8452-6.

Glerum, D. M. and A. Tzagoloff (1998). "Affinity purification of yeast cytochrome oxidase with biotinylated subunits 4, 5, or 6." Anal Biochem **260**(1): 38-43.

Glick, B. S. (1995). "Pathways and energetics of mitochondrial protein import in *Saccharomyces cerevisiae*." Methods Enzymol **260**: 224-31.

Granick, S. and S. I. Beale (1978). "Hemes, chlorophylls, and related compounds: biosynthesis and metabolic regulation." Adv Enzymol Relat Areas Mol Biol **46**: 33-203.

Hanna, M. G., I. P. Nelson, S. Rahman, R. J. Lane, J. Land, S. Heales, M. J. Cooper, A. H. Schapira, J. A. Morgan-Hughes and N. W. Wood (1998). "Cytochrome *c* oxidase deficiency associated with the first stop-codon point mutation in human mtDNA." Am J Hum Genet **63**(1): 29-36.

Heaton, D., T. Nittis, C. Srinivasan and D. R. Winge (2000). "Mutational analysis of the mitochondrial copper metallochaperone Cox17." J Biol Chem **275**(48): 37582-7.

Heaton, D. N., G. N. George, G. Garrison and D. R. Winge (2001). "The mitochondrial copper metallochaperone Cox17 exists as an oligomeric, polycopper complex." Biochemistry **40**(3): 743-51.

Hell, K., J. Herrmann, E. Pratje, W. Neupert and R. A. Stuart (1997). "Oxa1p mediates the export of the N- and C-termini of pCoxII from the mitochondrial matrix to the intermembrane space." FEBS Lett **418**(3): 367-70.

Hell, K., W. Neupert and R. A. Stuart (2001). "Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA." EMBO J **20**(6): 1281-8.

Hell, K., A. Tzagoloff, W. Neupert and R. A. Stuart (2000). "Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2." J Biol Chem **275**(7): 4571-8.

Hill, J. E., A. M. Myers, T. J. Koerner and A. Tzagoloff (1986). "Yeast/*E. coli* shuttle vectors with multiple unique restriction sites." Yeast **2**(3): 163-7.

Hiser, L. and J. P. Hosler (2001). "Heme A is not essential for assembly of the subunits of cytochrome *c* oxidase of *Rhodobacter sphaeroides*." J Biol Chem **276**(48): 45403-7.

Holt, I. J., A. E. Harding and J. A. Morgan-Hughes (1988). "Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies." Nature **331**(6158): 717-9.

Horng, Y. C., P. A. Cobine, A. B. Maxfield, H. S. Carr and D. R. Winge (2004). "Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome *c* oxidase." J Biol Chem.

Horvath, R., H. Lochmuller, R. Stucka, J. Yao, E. A. Shoubridge, S. H. Kim, K. D. Gerbitz and M. Jaksch (2000). "Characterization of human *SCO1* and *COX17* genes in mitochondrial cytochrome-*c*-oxidase deficiency." Biochem Biophys Res Commun **276**(2): 530-3.

Huttemann, M., B. Kadenbach and L. I. Grossman (2001). "Mammalian subunit IV isoforms of cytochrome c oxidase." Gene **267**(1): 111-23.

Huttemann, M., N. Muhlenbein, T. R. Schmidt, L. I. Grossman and B. Kadenbach (2000). "Isolation and sequence of the human cytochrome *c* oxidase subunit VIIaL gene." Biochim Biophys Acta **1492**(1): 252-8.

Huttemann, M., T. R. Schmidt and L. I. Grossman (2003). "A third isoform of cytochrome c oxidase subunit VIII is present in mammals." Gene **312**: 95-102.

Inoue, H., H. Nojima and H. Okayama (1990). "High efficiency transformation of *Escherichia coli* with plasmids." Gene **96**(1): 23-8.

Iwata, S., C. Ostermeier, B. Ludwig and H. Michel (1995). "Structure at 2.8 A resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*." Nature **376**(6542): 660-9.

Jaksch, M., R. Horvath, N. Horn, D. P. Auer, C. Macmillan, J. Peters, K. D. Gerbitz, I. Kraegeloh-Mann, A. Muntau, V. Karcagi, R. Kalmanchey, H. Lochmuller, E. A. Shoubridge and P. Freisinger (2001). "Homozygosity (E140K) in SCO2 causes delayed infantile onset of cardiomyopathy and neuropathy." Neurology **57**(8): 1440-6.

Jaksch, M., C. Paret, R. Stucka, N. Horn, J. Muller-Hocker, R. Horvath, N. Trepesch, G. Stecker, P. Freisinger, C. Thirion, J. Muller, R. Lunkwitz, G. Rodel, E. A. Shoubridge and H. Lochmuller (2001). "Cytochrome *c* oxidase deficiency due to mutations in *SCO2*, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts." Hum Mol Genet **10**(26): 3025-35.

Kako, K., K. Tsumori, Y. Ohmasa, Y. Takahashi and E. Munekata (2000). "The expression of Cox17p in rodent tissues and cells." Eur J Biochem **267**(22): 6699-707.

Karadimas, C. L., P. Greenstein, C. M. Sue, J. T. Joseph, K. Tanji, R. G. Haller, T. Taivassalo, M. M. Davidson, S. Shanske, E. Bonilla and S. DiMauro (2000). "Recurrent myoglobinuria due to a nonsense mutation in the *COX I* gene of mitochondrial DNA." Neurology **55**(5): 644-9.

Keightley, J. A., K. C. Hoffbuhr, M. D. Burton, V. M. Salas, W. S. Johnston, A. M. Penn, N. R. Buist and N. G. Kennaway (1996). "A microdeletion in cytochrome *c* oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria." Nat Genet **12**(4): 410-6.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(259): 680-5.

LaMarche, A. E., M. I. Abate, S. H. Chan and B. L. Trumpower (1992). "Isolation and characterization of *COX12*, the nuclear gene for a previously unrecognized subunit of Saccharomyces cerevisiae cytochrome c oxidase." J Biol Chem **267**(31): 22473-80.

Lange, H., A. Kaut, G. Kispal and R. Lill (2000). "A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins." Proc Natl Acad Sci U S A **97**(3): 1050-5.

Leary, S. C., B. A. Kaufman, G. Pellecchia, G. H. Guercin, A. Mattman, M. Jaksch and E. A. Shoubridge (2004). "Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome *c* oxidase." Hum Mol Genet **13**(17): 1839-48.

Lee, N., M. J. Daly, T. Delmonte, E. S. Lander, F. Xu, T. J. Hudson, G. A. Mitchell, C. C. Morin, B. H. Robinson and J. D. Rioux (2001). "A genomewide linkage-disequilibrium scan localizes the Saguenay-Lac-Saint-Jean cytochrome oxidase deficiency to 2p16." Am J Hum Genet **68**(2): 397-409.

Lode, A., M. Kuschel, C. Paret and G. Rodel (2000). "Mitochondrial copper metabolism in yeast: interaction between Sco1p and Cox2p." FEBS Lett **485**(1): 19-24.

Lode, A., C. Paret and G. Rodel (2002). "Molecular characterization of *Saccharomyces cerevisiae* Sco2p reveals a high degree of redundancy with Sco1p." Yeast **19**(11): 909-22.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall (1951). "Protein measurement with the Folin phenol reagent." J Biol Chem **193**(1): 265-75.

Manfredi, G., E. A. Schon, C. T. Moraes, E. Bonilla, G. T. Berry, J. T. Sladky and S. DiMauro (1995). "A new mutation associated with MELAS is located in a mitochondrial DNA polypeptide-coding gene." Neuromuscul Disord **5**(5): 391-8.

Manthey, G. M. and J. E. McEwen (1995). "The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial COX1 locus of Saccharomyces cerevisiae." EMBO J **14**(16): 4031-43.

Martin, R. G. and B. N. Ames (1961). "A method for determining the sedimentation behavior of enzymes: application to protein mixtures." J Biol Chem **236**: 1372-79.

Mashkevich, G., B. Repetto, D. M. Glerum, C. Jin and A. Tzagoloff (1997). "*SHY1*, the yeast homolog of the mammalian SURF-1 gene, encodes a mitochondrial protein required for respiration." J Biol Chem **272**(22): 14356-64.

Mattatall, N. R., J. Jazairi and B. C. Hill (2000). "Characterization of YpmQ, an accessory protein required for the expression of cytochrome *c* oxidase in *Bacillus subtilis*." J Biol Chem **275**(37): 28802-9.

Maxfield, A. B., D. N. Heaton and D. R. Winge (2004). "Cox17 is functional when tethered to the mitochondrial inner membrane." J Biol Chem **279**(7): 5072-80.

McEwen, J. E., C. Ko, B. Kloeckner-Gruissem and R. O. Poyton (1986). "Nuclear functions required for cytochrome *c* oxidase biogenesis in *Saccharomyces cerevisiae*. Characterization of mutants in 34 complementation groups." J Biol Chem **261**(25): 11872-9.

McMullin, T. W. and T. D. Fox (1993). "*COX3* mRNA-specific translational activator proteins are associated with the inner mitochondrial membrane in Saccharomyces cerevisiae." J Biol Chem **268**(16): 11737-41.

Merante, F., R. Petrova-Benedict, N. MacKay, G. Mitchell, M. Lambert, C. Morin, M. De Braekeleer, R. Laframboise, R. Gagne and B. H. Robinson (1993). "A biochemically distinct form of cytochrome oxidase (COX) deficiency in the Saguenay-Lac-Saint-Jean region of Quebec." Am J Hum Genet **53**(2): 481-7.

Michel, H., J. Behr, A. Harrenga and A. Kannt (1998). "Cytochrome *c* oxidase: structure and spectroscopy." Annu Rev Biophys Biomol Struct **27**: 329-56.

Mochizuki, M., H. Aoyama, K. Shinzawa-Itoh, T. Usui, T. Tsukihara and S. Yoshikawa (1999). "Quantitative reevaluation of the redox active sites of crystalline bovine heart cytochrome *c* oxidase." J Biol Chem **274**(47): 33403-11.

Mootha, V. K., P. Lepage, K. Miller, J. Bunkenborg, M. Reich, M. Hjerrild, T. Delmonte, A. Villeneuve, R. Sladek, F. Xu, G. A. Mitchell, C. Morin, M. Mann, T. J. Hudson, B. Robinson, J. D. Rioux and E. S. Lander (2003). "Identification of a gene causing human cytochrome *c* oxidase deficiency by integrative genomics." Proc Natl Acad Sci U S A **100**(2): 605-10.

Morin, C., G. Mitchell, J. Larochelle, M. Lambert, H. Ogier, B. H. Robinson and M. De Braekeleer (1993). "Clinical, metabolic, and genetic aspects of cytochrome *C* oxidase deficiency in Saguenay-Lac-Saint-Jean." Am J Hum Genet **53**(2): 488-96.

Mulero, J. J. and T. D. Fox (1993). "PET111 acts in the 5'-leader of the *Saccharomyces cerevisiae* mitochondrial *COX2* mRNA to promote its translation." Genetics **133**(3): 509-16.

Murakami, T., L. T. Reiter and J. R. Lupski (1997). "Genomic structure and expression of the human heme A:farnesyltransferase (*COX10*) gene." Genomics **42**(1): 161-4.

Nijtmans, L. G., M. Artal Sanz, M. Bucko, M. H. Farhoud, M. Feenstra, G. A. Hakkaart, M. Zeviani and L. A. Grivell (2001). "Shy1p occurs in a high molecular weight complex and is required for efficient assembly of cytochrome *c* oxidase in yeast." FEBS Lett **498**(1): 46-51.

Nijtmans, L. G., J. W. Taanman, A. O. Muijsers, D. Speijer and C. Van den Bogert (1998). "Assembly of cytochrome-*c* oxidase in cultured human cells." Eur J Biochem **254**(2): 389-94.

Nittis, T., G. N. George and D. R. Winge (2001). "Yeast Sco1, a protein essential for cytochrome *c* oxidase function is a Cu(I)-binding protein." J Biol Chem **276**(45): 42520-6.

Nobrega, M. P., S. C. Bandeira, J. Beers and A. Tzagoloff (2002). "Characterization of *COX19*, a widely distributed gene required for expression of mitochondrial cytochrome oxidase." J Biol Chem **277**(43): 40206-11.

Nobrega, M. P., F. G. Nobrega and A. Tzagoloff (1990). "*COX10* codes for a protein homologous to the ORF1 product of *Paracoccus denitrificans* and is required for the synthesis of yeast cytochrome oxidase." J Biol Chem **265**(24): 14220-6.

Oquendo, C. E., H. Antonicka, E. A. Shoubridge, W. Reardon and G. K. Brown (2004). "Functional and genetic studies demonstrate that mutation in the *COX15* gene can cause Leigh syndrome." J Med Genet **41**(7): 540-4.

Palumaa, P., L. Kangur, A. Voronova and R. Sillard (2004). "Metal-binding mechanism of Cox17, a copper chaperone for cytochrome *c* oxidase." Biochem J **382**(Pt 1): 307-14.

Papadopoulou, L. C., C. M. Sue, M. M. Davidson, K. Tanji, I. Nishino, J. E. Sadlock, S. Krishna, W. Walker, J. Selby, D. M. Glerum, R. V. Coster, G. Lyon, E. Scalais, R. Lebel, P. Kaplan, S. Shanske, D. C. De Vivo, E. Bonilla, M. Hirano, S. DiMauro and E. A. Schon (1999). "Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in *SCO2*, a COX assembly gene." Nat Genet **23**(3): 333-7.

Paret, C., K. Ostermann, U. Krause-Buchholz, A. Rentzsch and G. Rodel (1999). "Human members of the *SCO1* gene family: complementation analysis in yeast and intracellular localization." FEBS Lett **447**(1): 65-70.

Patterson, T. E. and R. O. Poyton (1986). "COX8, the structural gene for yeast cytochrome c oxidase subunit VIII. DNA sequence and gene disruption indicate that subunit VIII is required for maximal levels of cellular respiration and is derived from a precursor which is extended at both its NH2 and COOH termini." J Biol Chem **261**(36): 17192-7.

Pecina, P., M. Capkova, S. K. Chowdhury, Z. Drahota, A. Dubot, A. Vojtiskova, H. Hansikova, H. Houst'kova, J. Zeman, C. Godinot and J. Houstek (2003). "Functional alteration of cytochrome *c* oxidase by *SURF1* mutations in Leigh syndrome." Biochim Biophys Acta **1639**(1): 53-63.

Pecina, P., H. Houstkova, H. Hansikova, J. Zeman and J. Houstek (2004). "Genetic defects of cytochrome *c* oxidase assembly." Physiol Res **53 Suppl 1**: S213-23.

Pena, M. M., J. Lee and D. J. Thiele (1999). "A delicate balance: homeostatic control of copper uptake and distribution." J Nutr **129**(7): 1251-60.

Perez-Martinez, X., S. A. Broadley and T. D. Fox (2003). "Mss51p promotes mitochondrial Cox1p synthesis and interacts with newly synthesized Cox1p." Embo J **22**(21): 5951-61.

Peschek, G. A., D. Alge, S. Fromwald and B. Mayer (1995). "Transient accumulation of heme O (cytochrome o) in the cytoplasmic membrane of semi-anaerobic *Anacystis nidulans*. Evidence for oxygenase-catalyzed heme O/A transformation." J Biol Chem **270**(46): 27937-41.

Petruzzella, V., V. Tiranti, P. Fernandez, P. Ianna, R. Carrozzo and M. Zeviani (1998). "Identification and characterization of human cDNAs specific to *BCS1*, *PET112*, *SCO1*, *COX15*, and *COX11*, five genes involved in the formation and function of the mitochondrial respiratory chain." Genomics **54**(3): 494-504.

Porra, R. J., W. Schafer, E. Cmiel, I. Katheder and H. Scheer (1994). "The derivation of the formyl-group oxygen of chlorophyll b in higher plants from molecular oxygen. Achievement of high enrichment of the 7-formyl-group oxygen from $18O_2$ in greening maize leaves." Eur J Biochem **219**(1-2): 671-9.

Poyton, R. O. and J. E. McEwen (1996). "Crosstalk between nuclear and mitochondrial genomes." Annu Rev Biochem **65**: 563-607.

Punter, F. A., D. L. Adams and D. M. Glerum (2000). "Characterization and localization of human *COX17*, a gene involved in mitochondrial copper transport." Hum Genet **107**(1): 69-74.

Punter, F. A. and D. M. Glerum (2003). "Mutagenesis reveals a specific role for Cox17p in copper transport to cytochrome oxidase." J Biol Chem **278**(33): 30875-80.

Puustinen, A. and M. Wikstrom (1991). "The heme groups of cytochrome o from Escherichia coli." Proc Natl Acad Sci U S A **88**(14): 6122-6.

Rahman, S., J. W. Taanman, J. M. Cooper, I. Nelson, I. Hargreaves, B. Meunier, M. G. Hanna, J. J. Garcia, R. A. Capaldi, B. D. Lake, J. V. Leonard and A. H. Schapira (1999). "A missense mutation of cytochrome oxidase subunit II causes defective assembly and myopathy." Am J Hum Genet **65**(4): 1030-9.

Rentzsch, A., G. Krummeck-Weiss, A. Hofer, A. Bartuschka, K. Ostermann and G. Rodel (1999). "Mitochondrial copper metabolism in yeast: mutational analysis of Sco1p involved in the biogenesis of cytochrome *c* oxidase." Curr Genet **35**(2): 103-8.

Rep, M. and L. A. Grivell (1996). "The role of protein degradation in mitochondrial function and biogenesis." Curr Genet **30**(5): 367-80.

Saiki, K., T. Mogi and Y. Anraku (1992). "Heme O biosynthesis in *Escherichia coli*: the *cyoE* gene in the cytochrome *bo* operon encodes a protoheme IX farnesyltransferase." Biochem Biophys Res Commun **189**(3): 1491-7.

Salviati, L., E. Hernandez-Rosa, W. F. Walker, S. Sacconi, S. DiMauro, E. A. Schon and M. M. Davidson (2002). "Copper supplementation restores cytochrome *c* oxidase activity in cultured cells from patients with *SCO2* mutations." Biochem J **363**(Pt 2): 321-7.

Saracco, S. A. and T. D. Fox (2002). "Cox18p is required for export of the mitochondrially encoded *Saccharomyces cerevisiae* Cox2p C-tail and interacts with Pnt1p and Mss2p in the inner membrane." Mol Biol Cell **13**(4): 1122-31.

Schagger, H. and G. von Jagow (1987). "Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa." Anal Biochem **166**(2): 368-79.

Schiestl, R. H. and R. D. Gietz (1989). "High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier." Curr Genet **16**(5-6): 339-46.

Seraphin, B., M. Simon and G. Faye (1988). "*MSS18*, a yeast nuclear gene involved in the splicing of intron aI5 beta of the mitochondrial *cox1* transcript." Embo J **7**(5): 1455-64.

Shoubridge, E. A. (2001). "Cytochrome *c* oxidase deficiency." Am J Med Genet **106**(1): 46-52.

Simon, M., B. Seraphin and G. Faye (1995). "The nuclear-encoded *MSS2* gene is involved in the expression of the mitochondrial cytochrome-*c* oxidase subunit 2 (Cox2)." Biochim Biophys Acta **1228**(1): 95-8.

Smeitink, J., L. van den Heuvel and S. DiMauro (2001). "The genetics and pathology of oxidative phosphorylation." Nat Rev Genet **2**(5): 342-52.

Srinivasan, C., M. C. Posewitz, G. N. George and D. R. Winge (1998). "Characterization of the copper chaperone Cox17 of *Saccharomyces cerevisiae*." Biochemistry **37**(20): 7572-7.

Stuart, R. (2002). "Insertion of proteins into the inner membrane of mitochondria: the role of the Oxa1 complex." Biochim Biophys Acta **1592**(1): 79-87.

Svensson, B., K. K. Andersson and L. Hederstedt (1996). "Low-spin heme A in the heme A biosynthetic protein CtaA from Bacillus subtilis." Eur J Biochem **238**(1): 287-95.

Svensson, B. and L. Hederstedt (1994). "*Bacillus subtilis* CtaA is a heme-containing membrane protein involved in heme A biosynthesis." J Bacteriol **176**(21): 6663-71.

Svensson, B., M. Lubben and L. Hederstedt (1993). "Bacillus subtilis CtaA and CtaB function in haem A biosynthesis." Mol Microbiol **10**(1): 193-201.

Taanman, J. W. (1999). "The mitochondrial genome: structure, transcription, translation and replication." Biochim Biophys Acta **1410**(2): 103-23.

Taanman, J. W. and R. A. Capaldi (1992). "Purification of yeast cytochrome *c* oxidase with a subunit composition resembling the mammalian enzyme." J Biol Chem **267**(31): 22481-5.

Taanman, J. W. and R. A. Capaldi (1993). "Subunit VIa of yeast cytochrome *c* oxidase is not necessary for assembly of the enzyme complex but modulates the enzyme activity. Isolation and characterization of the nuclear-coded gene." J Biol Chem **268**(25): 18754-61.

Taanman, J. W. and S. L. Williams (2001). "Assembly of cytochrome *c* oxidase: what can we learn from patients with cytochrome *c* oxidase deficiency?" Biochem Soc Trans **29**(Pt 4): 446-51.

Takahashi, Y., K. Kako, S. Kashiwabara, A. Takehara, Y. Inada, H. Arai, K. Nakada, H. Kodama, J. Hayashi, T. Baba and E. Munekata (2002). "Mammalian copper chaperone

Cox17p has an essential role in activation of cytochrome *C* oxidase and embryonic development." Mol Cell Biol **22**(21): 7614-21.

Takahashi, Y., K. Kako, K. Ohmura, K. Tsumori, Y. Ohmasa, S. Kashiwabara, T. Baba and E. Munekatat (2001). "Genomic structure of mouse copper chaperone, *COX17*." DNA Seq **12**(5-6): 305-18.

Tiranti, V., P. Corona, M. Greco, J. W. Taanman, F. Carrara, E. Lamantea, L. Nijtmans, G. Uziel and M. Zeviani (2000). "A novel frameshift mutation of the mtDNA COIII gene leads to impaired assembly of cytochrome *c* oxidase in a patient affected by Leigh-like syndrome." Hum Mol Genet **9**(18): 2733-42.

Tiranti, V., C. Galimberti, L. Nijtmans, S. Bovolenta, M. P. Perini and M. Zeviani (1999). "Characterization of *SURF-1* expression and Surf-1p function in normal and disease conditions." Hum Mol Genet **8**(13): 2533-40.

Tiranti, V., M. Jaksch, S. Hofmann, C. Galimberti, K. Hoertnagel, L. Lulli, P. Freisinger, L. Bindoff, K. D. Gerbitz, G. P. Comi, G. Uziel, M. Zeviani and T. Meitinger (1999). "Loss-of-function mutations of *SURF-1* are specifically associated with Leigh syndrome with cytochrome *c* oxidase deficiency." Ann Neurol **46**(2): 161-6.

Tsukihara, T., H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono and S. Yoshikawa (1995). "Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å." Science **269**(5227): 1069-74.

Tsukihara, T., H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono and S. Yoshikawa (1996). "The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 A." Science **272**(5265): 1136-44.

Tsukihara, T., K. Itoh-Shinzawa and S. Yoshikawa (1996). "[Structures of metal centers of bovine heart cytochrome *c* oxidase]." Tanpakushitsu Kakusan Koso **41**(9): 1353-62.

Tsukihara, T. and S. Yoshikawa (1998). "Crystal structural studies of a membrane protein complex, cytochrome *c* oxidase from bovine heart." Acta Crystallogr A **54**(Pt 6 Pt 1): 895-904.

Tzagoloff, A., N. Capitanio, M. P. Nobrega and D. Gatti (1990). "Cytochrome oxidase assembly in yeast requires the product of *COX11*, a homolog of the *P. denitrificans* protein encoded by ORF3." EMBO J **9**(9): 2759-64.

Tzagoloff, A. and C. L. Dieckmann (1990). "PET genes of *Saccharomyces cerevisiae*." Microbiol Rev **54**(3): 211-25.

Tzagoloff, A., M. Nobrega, N. Gorman and P. Sinclair (1993). "On the functions of the yeast *COX10* and *COX11* gene products." Biochem Mol Biol Int **31**(3): 593-8.

Valencik, M. L., B. Kloeckener-Gruissem, R. O. Poyton and J. E. McEwen (1989). "Disruption of the yeast nuclear *PET54* gene blocks excision of mitochondrial intron aI5 beta from pre-mRNA for cytochrome *c* oxidase subunit I." Embo J **8**(12): 3899-904.

Valencik, M. L. and J. E. McEwen (1991). "Genetic evidence that different functional domains of the *PET54* gene product facilitate expression of the mitochondrial genes *COX1* and *COX3* in *Saccharomyces cerevisiae*." Mol Cell Biol **11**(5): 2399-405.

Valnot, I., S. Osmond, N. Gigarel, B. Mehaye, J. Amiel, V. Cormier-Daire, A. Munnich, J. P. Bonnefont, P. Rustin and A. Rotig (2000). "Mutations of the *SCO1* gene in mitochondrial cytochrome *c* oxidase deficiency with neonatal-onset hepatic failure and encephalopathy." Am J Hum Genet **67**(5): 1104-9.

Valnot, I., J. C. von Kleist-Retzow, A. Barrientos, M. Gorbatyuk, J. W. Taanman, B. Mehaye, P. Rustin, A. Tzagoloff, A. Munnich and A. Rotig (2000). "A mutation in the human heme A:farnesyltransferase gene (*COX10*) causes cytochrome *c* oxidase deficiency." Hum Mol Genet **9**(8): 1245-9.

Von Kleist-Retzow, J. C., J. Yao, J. W. Taanman, K. Chantrel, D. Chretien, V. Cormier-Daire, A. Rotig, A. Munnich, P. Rustin and E. A. Shoubridge (2001). "Mutations in *SURF1* are not specifically associated with Leigh syndrome." J Med Genet **38**(2): 109-13.

Wallace, D. C., G. Singh, M. T. Lott, J. A. Hodge, T. G. Schurr, A. M. Lezza, L. J. Elsas, 2nd and E. K. Nikoskelainen (1988). "Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy." Science **242**(4884): 1427-30.

Wallace, D. C., X. X. Zheng, M. T. Lott, J. M. Shoffner, J. A. Hodge, R. I. Kelley, C. M. Epstein and L. C. Hopkins (1988). "Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease." Cell **55**(4): 601-10.

Wielburski, A. and B. D. Nelson (1983). "Evidence for the sequential assembly of cytochrome oxidase subunits in rat liver mitochondria." Biochem J **212**(3): 829-34.

Wielburski, A. and B. D. Nelson (1984). "Heme a induces assembly of rat liver cytochrome *c* oxidase subunits I--III in isolated mitochondria." FEBS Lett **177**(2): 291-4.

Williams, S. L., I. Valnot, P. Rustin and J. W. Taanman (2004). "Cytochrome *c* oxidase subassemblies in fibroblast cultures from patients carrying mutations in *COX10*, *SCO1*, or *SURF1*." J Biol Chem **279**(9): 7462-9.

Winge, D. R., L. T. Jensen and C. Srinivasan (1998). "Metal-ion regulation of gene expression in yeast." Curr Opin Chem Biol **2**(2): 216-21.

Xu, F., C. Morin, G. Mitchell, C. Ackerley and B. H. Robinson (2004). "The role of the LRPPRC (leucine-rich pentatricopeptide repeat cassette) gene in cytochrome oxidase

assembly: mutation causes lowered levels of COX (cytochrome *c* oxidase) I and COX III mRNA." Biochem J **382**(Pt 1): 331-6.

Yao, J. and E. A. Shoubridge (1999). "Expression and functional analysis of SURF1 in Leigh syndrome patients with cytochrome *c* oxidase deficiency." Hum Mol Genet **8**(13): 2541-9.

Yon, J., T. Jones, K. Garson, D. Sheer and M. Fried (1993). "The organization and conservation of the human *Surfeit* gene cluster and its localization telomeric to the c-abl and can proto-oncogenes at chromosome band 9q34.1." Hum Mol Genet **2**(3): 237-40.

Yoshikawa, S., K. Shinzawa-Itoh and T. Tsukihara (1998). "Crystal structure of bovine heart cytochrome *c* oxidase at 2.8 A resolution." J Bioenerg Biomembr **30**(1): 7-14.

Zhang, Q. H., M. Ye, X. Y. Wu, S. X. Ren, M. Zhao, C. J. Zhao, G. Fu, Y. Shen, H. Y. Fan, G. Lu, M. Zhong, X. R. Xu, Z. G. Han, J. W. Zhang, J. Tao, Q. H. Huang, J. Zhou, G. X. Hu, J. Gu, S. J. Chen and Z. Chen (2000). "Cloning and functional analysis of cDNAs with open reading frames for 300 previously undefined genes expressed in CD34+ hematopoietic stem/progenitor cells." Genome Res **10**(10): 1546-60.

Zhu, Z., J. Yao, T. Johns, K. Fu, I. De Bie, C. Macmillan, A. P. Cuthbert, R. F. Newbold, J. Wang, M. Chevrette, G. K. Brown, R. M. Brown and E. A. Shoubridge (1998). "*SURF1*, encoding a factor involved in the biogenesis of cytochrome *c* oxidase, is mutated in Leigh syndrome." Nat Genet **20**(4): 337-43.